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ANALYSIS OF CHROMATIN ACCESSIBILITY OF THE HUMAN C-MYC
REPLICATION ORIGIN

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

TU DANH
B.S., Calvin College, 2011

2015
Wright State University

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Tu Thien Danh

2015

WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

November 9, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY Tu Danh ENTITLED Analysis Of Chromatin Accesibility Of The
Human C-Myc Origin BE ACCEPTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Danh, Tu M.S. Department of Biochemistry and Molecular Biology M.S. Program, Wright State University, 2015. Analysis of chromatin accessibility of the human c-myc replication origin.

The best characterized eukaryote replication model is of the budding yeast *Saccharomyces cerevisiae*. Replication origins of *S.cerevisiae* are 100 to 200 bp in size and contain an essential 11-bp autonomous replicating sequence (ARS) consensus sequence (ACS). The origin recognition complex (ORC) binds to the ACS in order to recruit additional replication factors (Cdt1, Cdc6, MCM, Cdc45) and together they form the pre-replication complex (pre-RC).

Unlike budding yeast, the mammalian cells contain dispersed replication origins in which multiple elements distributed over large distances act as replication start sites. Mammalian DNA replication origins, such as the c-myc origin, contain a DNA unwinding element (DUE), which is an AT-rich region that contains 10-of-11 matches to ARS consensus. Our lab primarily studies DNA replication of the human c-myc locus. We have successfully integrated the wild-type 2.4 kb c-myc replication origin and its various inactive mutants in a known ectopic chromosomal site in HeLa/406 cells in order to study the multiple functional elements of this origin.

My thesis focused on investigating the minimal sequence required in mammalian replication origin for replication activity. To address this question, I assessed the effects of replication protein tethering on chromatin accessibility of inactivated c-myc origins which are 930 bp or less and contain an intact DUE. In part I of my thesis, I describe how I made two new HeLa/406 cell lines containing two different deletion mutants of the c-myc replicator core (which are 607 bp and 228 bp long) at the known ectopic chromosomal site. In part II of my thesis, I describe how I created plasmids expressing GAL4 DNA-binding domain (GAL4^{DBD})-BRG1 fusion proteins. BRG1 is a catalytic core

subunit of SWI/SNF mammalian chromatin remodeling complex. Wild-type BRG1 and catalytic inactive BRG1^{K798R} mutant were used to make GAL4^{DBD} fusion proteins.

In part III of my thesis, I evaluated the effects of GAL4^{DBD} fusion proteins on chromatin structure of the inactivated ectopic c-myc replicator via DNase I hypersensitivity assay. The proteins used were Cdt1, a licensing protein necessary for pre-RC formation; wild-type BRG1 and catalytically dead BRG1 mutant. GAL4^{DBD}-Cdt1 has previously been shown to restore origin activity, as well as binding of replication proteins such as ORC, MCM, Cdc45, and DUE-B. My data suggest that expression of GAL4^{DBD}-Cdt1 or of GAL4^{DBD}-wild-type BRG1 can significantly increase the DNase I sensitivity of the inactivated ectopic 5' 930 bp c-myc origin, which contains both the DUE and the GAL4 binding sites. However, the shorter c-myc replicators (607 bp or 228 bp) did not show any change in DNase I hypersensitivity when the same GAL4^{DBD}-Cdt1 was expressed. These results indicate that in conjunction with GAL4^{DBD} protein tethering, the c-myc DUE alone is not sufficient for opening the chromatin structure of the ectopic c-myc replicator. Thus, the reactivation of the ectopic c-myc replicator via GAL4^{DBD} protein tethering requires additional DNA elements besides the DUE.

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I. INTRODUCTION

DNA replication and DNA unwinding:

Chromosomes are the permanent archives of the information which directs cell function. Thus, duplication of eukaryotic chromosomes is an essential part of the cell cycle. This process is tightly regulated throughout the G1 and S phases, because any errors (such as incompleteness, inaccuracies, excessive DNA synthesis, etc.) can lead to dysfunctions such as cell death, developmental defects, and cancer transformation. Before beginning the process of DNA replication in S phase, the two DNA templates are first unwound by DNA helicases (collectively known as the Cdc45-Mcm2-7-GINS or CMG complex) and DNA polymerases are recruited to the same complex (Masai et al., 2011).

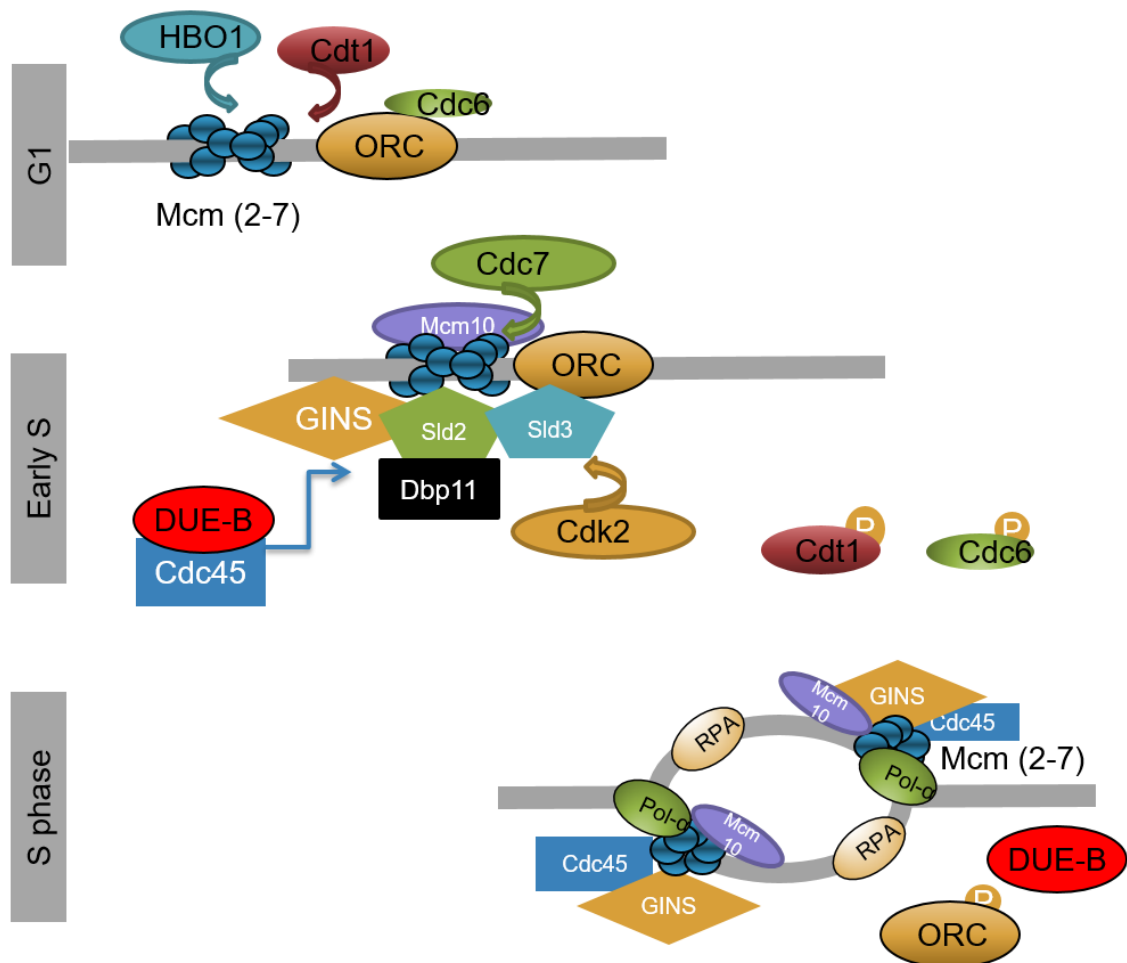
The stepwise assembly of the initiation complex begins in G1 and continues to early S phase (Figure 1). In G1 phase, chromatin structure of origin DNA is decondensed in order to allow accessibility of helicases and other replication proteins. HBO1, which form a complex with licensing factor Cdt1, is an acetyltransferase which causes histone H4 acetylation, thus loosening nucleosomal packing (Miotto and Struhl, 2010). As a result of chromatin modification, DNA helicase Mcm2-7 complex can be loaded onto replication origins with the help of Cdt1, Cdc6, and origin-recognition complex (ORC, which contains Orc1-Orc6 proteins) (Bell and Dutta, 2002). Together, they form the pre-replication complex (pre-RC), which remains inactive until the early onset of S phase, in which the cyclin-dependent kinase (CDK) level rises.

In *S.cerevisiae*, the Dbf4-dependent Cdc7 kinase (DDK) phosphorylates Mcm2-7 helicase so that Sld3 can be loaded onto the pre-RC. Cdk2 (CDK) phosphorylates the replication factors Sld3 and Sld2. Subsequently, these phosphorylated factors can interact with Dbp11 or Cut5 (*S.cerevisiae* or *S.pombe* orthologs of human TopBP1) to recruit helicase activators Cdc45 and GINS to Mcm 2-7 complex (Zegerman and Diffley 2007). Together, Cdc45, GINS and the double hexameric Mcm2-7 complex form the CMG

helicase. These replication factors (Sld2, Sld3, Dbp11/Cut5) dissociate after the CMG assembly, which indicates that the involvement of said factors is likely to be minimal after the completion of this process. DUE-B (DNA unwinding element binding protein) has been shown to interact with Cdc45 at active replication origin. Additionally, phosphorylation of DUE-B C-terminus regulates DUE-B interaction during DNA replication initiation (Yan et al., 2014). In *Xenopus* egg extract, phosphorylated DUE-B forms homodimers, whereas unphosphorylated DUE-B forms dimers as well as high molecular weight complex (HMW) with Mcm2-7 helicases. During G1/S phase, Mcm2-7 is released from HMW by Cdc7 kinase activity from the HMW complex, and DUE-B is phosphorylated. Conversely, DUE-B is unphosphorylated in M phase by protein phosphatase PP2A and the HMW complex with Mcm2-7 is formed. These results are consistent with previous studies which show that the equilibrium between Cdc7 and PP2A activity is important for cell cycle transitions as well as DNA replication initiation (Heller et al., 2011; Krasinka et al., 2011; and Chou et al., 2002).

Once the CMG complex is formed, Mcm2-7 helicase can unwind double stranded DNA. RPA (Replication Protein A) is recruited to the origin to bind the single-strand unwound DNA (ssDNA), polymerase α (Pol α) generate DNA primer to initiate DNA synthesis on both strands (Burgers 2009). Finally, polymerases ϵ and δ , responsible for DNA replication of leading (pol ϵ) and lagging (pol δ) strands, are recruited to the origin, thereby initiating DNA synthesis (Larrea et al., 2010; Nick McElhinny et al., 2008; and Pursell et al., 2007). These results, however, contradict with a recent genetic study of *S.cerevisiae*, which showed that DNA pol δ plays a major role in replication of both leading and lagging DNA strands (Johnson et al., 2015). In its entirety, the protein complex (CMG, polymerases α , ϵ , δ and RPA) formed after the recruitment of DNA polymerases is called the replisome. Mcm10 is another important protein for DNA replication initiation which has been shown to bind both dsDNA and ssDNA in vitro (Fien et al., 2004). Mcm10 can interact with PCNA (a processivity factor of DNA replication) and Pol α . Moreover, reduced pol α and pol δ loadings in the absence of Mcm10 have been shown both in vitro (Heller et al., 2011) and in vivo (Kanke et al., 2012). These data, taken together, suggest that Mcm10 acts as a scaffolding protein for the replisome as the replication fork moves bidirectionally.

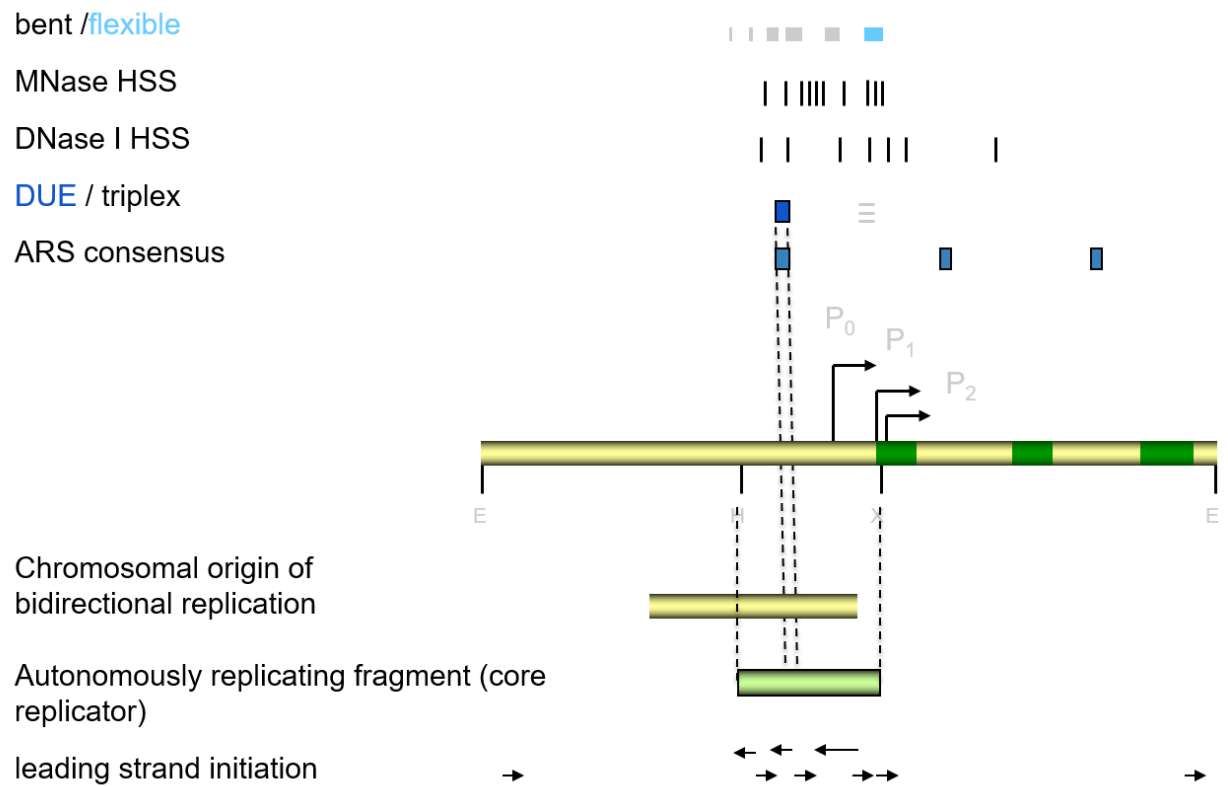
Figure 1. Stepwise Assembly of the CMG helicases before DNA replication initiation in eukaryotic cells.



Human c-myc replication origin:

The human-c-myc locus contains a chromosomal origin of bidirectional replication (Figure 2). This origin has 2.4 kb c-myc core replicator, which comprises both replicator elements and replication start sites. The c-myc core replicator is an autonomously replicating fragment, which retains its replication activity after being integrated into a different chromosomal location (Malott and Leffak, 1999; Liu et al., 2003). Among the structural elements of the 2.4 kb c-myc origin core are the DNA unwinding element (DUE), a series of positioned nucleosomes, several micrococcal nuclease hypersensitive and DNase I hypersensitive sites, and several DNA segments that are bent or in flexible conformations *in vitro* (Figure 2) (Liu et al., 2003). The DUE, which contains three 10-of-11 matches to the *S.cerevisiae* ARS consensus, is A-T rich and predicted to be unwound *in vivo*. Furthermore, it has been shown that DUE is essential for replicator activity and is a target of DUE-B protein *in vivo* (Liu et al., 2003; Casper et al., 2005; Kemp et al., 2007). Thus, the presence of these structures suggests that the chromatin of c-myc origin core is in an open confirmation for DNA unwinding and DNA replication.

Figure 2. Human c-myc locus (Chen, 2011)



Activation of human chromosomal replication origin via GAL4 protein tethering:

In order to model the effect of chromatin acetylation, replication protein binding, and transcription on a specific replication origin, constructs of the c-myc core replicator and its mutants have been successfully integrated by FLP recombinase at an ectopic chromosomal site as a single copy. Using this system, the ectopic 2.4 kb c-myc replicator was shown to have full bidirectional replication activity, nucleosomal positioning and chromatin structures of the endogenous c-myc origin (Liu et al., 2003; Ghosh et al., 2006). It has also been shown that the binding of replication proteins such as the ORC, Mcm2-7, Cdc45 and DUE-B via chromatin immunoprecipitation (ChIP) is comparable to that of the endogenous c-myc origin (Ghosh et al., 2006; Liu et al., 2012).

Furthermore, when the 3' 1.4 kb c-myc DNA of the 2.4 kb c-myc replicator was replaced by a cassette containing five tandem GAL4-binding sites (5xGAL4), the ectopic c-myc replicator (also known as FRT.myc.5'(930)-GAL4) was inactivated. Because of this 5xGAL4 replacement, several replication proteins (HBO1, Orc2, and Cdt1), along with transcription factors E2F1 and CREB, can be targeted to the inactivated ectopic c-myc replicator by fusing the GAL4 DNA-binding domain (GAL4^{DBD}) with these proteins (Chen et al., 2013). As a result, the endogenous replication proteins such as Mcm2-7, DUE-B, and Cdc45 were recruited to the inactivated c-myc origin, and origin activity was fully restored to that of the 2.4 kb c-myc replicator. Consistently, the c-myc origin DNA shows enhanced local chromatin acetylation in the presence of acetyltransferase HBO1-GAL4^{DBD}, which is suggestive of an open chromatin structure.

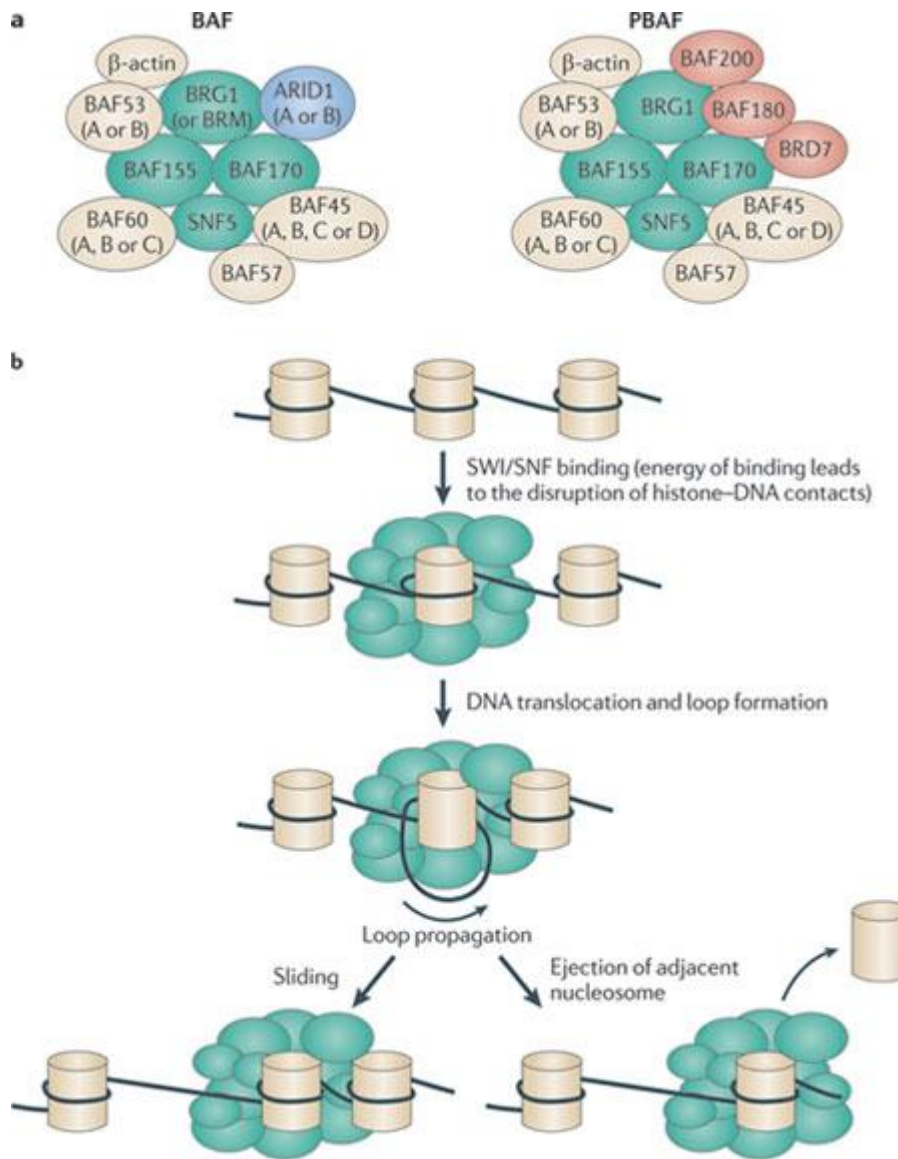
The catalytic core subunit BRG1/SMARCA4 of human SWI/SNF chromatin remodeling complex:

Human SWI/SNF complex is a multi-subunit assembly, which regulates transcription by altering chromatin structures. The complexes are composed of highly conserved core subunits (green) and variant subunits (yellow) (Figure 3a). The mammalian complexes have either BRM/SMARCA2 (Brahma) or BRG1/SMARCA4 (Brahma-related gene 1), which are mutually exclusive ATPase core subunits. The stepwise process of chromatin remodeling is depicted in Figure 3b. In an inactive gene region, chromatin is condensed and DNA is tightly wrapped around nucleosomes.

SWI/SNF can bind the chromatin and utilize the energy derived from ATP hydrolysis to destabilize histone-DNA contact and form a free DNA loop. Once the DNA loop is propagated, the complex can either slide to the nucleosome position or eject the adjacent nucleosome. As a result, the DNA is free from the nucleosome, which leads to the increased accessibility of DNA-binding factors to their targets (Côté et al., 1994).

To assess whether opening the chromatin structure of the inactivated FRT.myc.5'(930)-GAL4 replicator plays a role in restored origin activity, we asked whether the expression of either GAL4^{DBD}-Cdt1 or GAL4^{DBD}-BRG1 fusion proteins can increase the DNase I hypersensitivity of this c-myc replicator. We also asked whether the same GAL4^{DBD} fusion proteins can affect the chromatin structure of two new shortened c-myc replicators (607 bp and 228 bp), both of which have not only an intact DUE but also GAL4 binding sites.

Figure 3: The composition and mechanism of SWI/SNF complexes (Wilson et al., 2011)



II. MATERIAL AND METHODS

Construction of plasmids:

Plasmids expressing GAL4^{DBD} fusion proteins:

Plasmids pBABE BRG1, BRG1^{K798R} were generous gifts from Dr. Ivana de la Serna. Using In-Fusion HD cloning kit (Clontech), these cDNAs were cloned into a linearized pEGFP GAL4^{DBD}, which has the GAL4^{DBD} in place of EGFP cDNA. The genes of interest (BRG1, BRG1^{K798R}) were amplified with PCR primers engineered with 15 bp extensions homologous to vector ends. After the sizes of PCR products were verified on 1% agarose gel electrophoresis, the PCR products were purified using E.Z.N.A cycle pure kit (Omega Biotek). The purified PCR products were incubated with the linearized vector (via SalI and MfeI sites) in In-Fusion enzyme premix, for 15 minutes at 50°C. The cloning reaction was transformed into Stellar Competent cells (Clontech), as recommended by the manufacturer's instructions. The screened clones were confirmed by restriction digests and DNA sequencing.

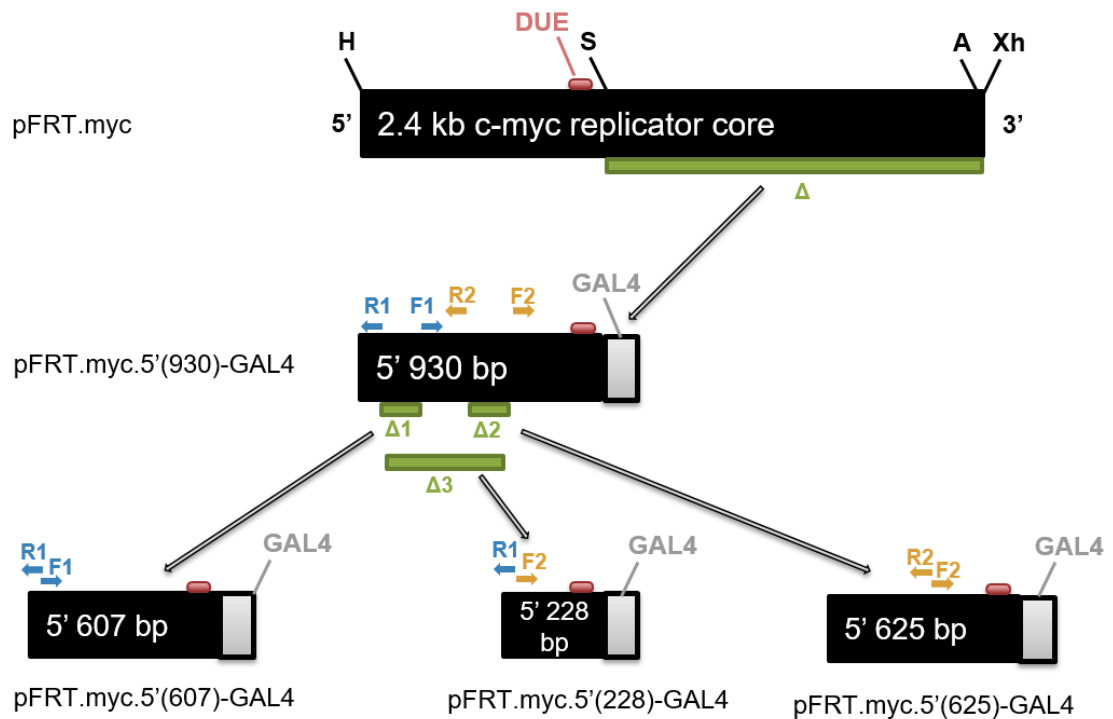
To select for BRG1 expression in blasticidin-treated HeLa cells, the blasticidin S deaminase (BSD) cDNA was added to the plasmid containing the GAL4^{DBD}-BRG1/BRG1^{K798R} cDNA. The pEGFP GAL4^{DBD}-BRG1/BRG1^{K798R} DNA constructs were cloned into pZFN-BSD by replacing the zinc-finger nuclease (ZFN) cDNA. The pZFN-BSD vector was linearized at NheI and PmeI sites. The GAL4^{DBD}-BRG1/BRG1^{K798R} was digested by NheI and PsiI, with PsiI producing a blunt end and NheI producing a sticky end. The ligation reaction was catalyzed by T4 DNA Ligase (New England Biolabs), incubated at 16°C overnight, and transformed into *E.coli* DH5α competent cells. The structure of plasmid containing GAL4^{DBD}-BRG1-BSD was confirmed via restriction digests and DNA sequencing.

Plasmids for cell line construction:

Plasmid pFRT.myc contains the full length 2.4 kb c-myc replicator core. When integrated in a known chromosomal location in HeLa/406 cells, this c-myc origin has been shown to have full replication activity *in vivo* (Chen et al., 2013). On the contrary, the plasmid pFRT.myc.5'(930)-GAL4 contains the inactivated c-myc replicator, which has a 3' 1420 bp DNA sequence replaced by five tandem GAL-4 binding sites (5xGAL4). After diagnostic digestion with PstI, this plasmid DNA was used as a template for inverse PCR, using three sets of phosphorylated primers, as depicted in Figure 4 (primer F1-R1 set, primer F2-R2 set, primer F2-R1 set). The PCR products were a linearized version of three new c-myc replicator mutants containing an intact DUE sequence, and, respectively, 607, 625, and 228 bp of c-myc DNA. The PCR products were purified and quantified using 1% agarose gel electrophoresis, followed by the ligation reaction using T4 DNase ligase. The newly constructed deletion constructs were verified via diagnostic digestion with PstI and DNA sequencing. The new plasmids were named according to their length of c-myc DNA: pFRT.myc.5'(607)-GAL4, pFRT.myc.5'(625)-GAL4, and pFRT.myc.5'(228)-GAL4.

Figure 4. DNA maps of c-myc replicators used in this study.

The HindIII-XhoI fragment contains the wild-type 2.4 kb c-myc replicator core, of which the 5' and 3' ends are shown in the map of pFRT.myc (A, ApaI; H, HindIII; S, SpeI; Xh; XhoI). The *in vivo* DNA unwinding element (DUE) is indicated in red. The 3' 1420 bp of the c-myc core origin (indicated by the Δ green box) was replaced by five tandem GAL4 binding sites (grey) to construct pFRT.myc.5'(930)-GAL4. Blue primer pair (forward F1 and reverse R1) and gold primer pair (forward F2 and reverse R2) are phosphorylated inverse PCR primers. These primers hybridized in an outward direction, and the PCR products had phosphorylated ends which can be ligated to produce circular DNA constructs. Thus, the final constructs would be lacking the DNA sequences flanked by their respective primer set (F1-R1, F2-R2, F2-R1), indicated by the green boxes ($\Delta 1$, $\Delta 2$, and $\Delta 1$). The new constructs are mapped as pFRT.myc.5'(607)-GAL4, pFRT.myc.5'(625)-GAL4 and pFRT.myc.5'(228)-GAL4.



Western blotting and antibodies

Protein expression was determined in cell lysates by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by western blotting. M-PER mammalian protein extraction reagent (Thermo Scientific) and proteinase inhibitor (20 µg/mL, Sigma-Aldrich) were used as cell lysis buffer. After electrophoresis, proteins were transferred to PVDF membrane (Immobilon-P transfer Millipore) by Genie Blotter (Idea Scientific) for 60 minutes, at constant current, 0.90 mA. Anti-β-actin and anti-FLAG antibodies were purchased from Sigma-Aldrich.

Cell line construction

Approximately 3.0×10^5 HeLa/406 cells, containing one FLP recombinase target (FRT) site at a specific chromosomal location (Liu et al., 2003), were plated in individual wells of a 24-well plate in Dulbecco's Modified Eagle Medium (4.5 g/L Glucose, L-Glutamine) with 10% newborn calf serum and incubated in 5% CO₂ at 37°C. Once the wells were 90-95% confluent, 0.8 µg of pFRT.myc donor plasmid, 0.1 µg of FLP recombinase expressing plasmid pOG44 and 2 µL of Lipofectamine 2000 were used for transfection per well. 24 hours after transfection, the cells were trypsinized, transferred from one well into one 15 cm diameter plate, in fresh media containing 600 µg/mL G418. The cells were maintained by re-plating in this fashion every third day.

After 14 days of monitoring the cells, the cells were then maintained in media containing 20 µM ganciclovir (GCV) for two days, followed by media containing 400 µg/mL G418 for three days, followed by media containing 20 µM GCV for two days. If the cells had successful integration of the pFRT.myc donor plasmid, the HSV thymidine kinase gene is interrupted, which subsequently renders the cells resistant to GCV selection. After the GCV selection, the single surviving mother colonies were transferred via cloning discs into individual wells of a 24-well plate of fresh media containing no antibiotic. Once the cells became confluent, they were re-plated out on a 10 cm plate in 10 mL of media containing 20 µM GCV for two days, followed by media containing 400 µg/mL G418 for three days. This was the last drug selection before getting the cells ready for PCR screening.

The cells were collected at 95% confluence. Half of the cells were frozen in liquid N₂, and half was used for genomic DNA extraction. A total of 50 ng of genomic DNA was used for PCR with HotStar Taq DNA polymerase (QIAGEN) and primer sets specific for either the unoccupied construct (primers 1 and 2) or the occupied construct after successful integration of the c-myc replicators (primers 1 and 3).

DNAse hypersensitivity assay

HeLa/406 derivative cell lines containing the c-myc replicator integrated at the ectopic FRT site were cultured in DMEM with 10% newborn calf serum in 5% CO₂ at 37°C. Approximately 1.0 x 10⁶ cells in each well per 6-well plate were transfected with 8 µg of GFP-N1 control plasmid, or GAL4-fusion protein expression plasmids or no DNA. A mixture of 10 µL Lipofectamine 2000 (Invitrogen) mixed with 250 µL Opti-MEM reduced serum medium was used for each plasmid DNA transfection, as recommended by the transfection manual. The GAL4-fusion plasmids used in this study are GAL4-Cdt1, GAL4-BRG1 wild-type and K798R mutant. Cells were trypsinized and harvested 24 hours after GAL4-Cdt1 transfection. Cells were treated with 10 µg/mL blasticidin 24 hours after GAL-BRG1 transfection, followed by harvesting 24 hours after. All cell lines used in this assay are: HeLa/FRT.myc.5'(930), HeLa/FRT.myc.5'(625)-GAL4, HeLa/FRT.myc.5'(607)-GAL4 and HeLa/FRT.myc.5'(228)-GAL4.

The cells were spun down at 500 g for 1 minute, washed with ice-cold phosphate buffered saline (PBS). The cell pellets were washed two more times, and then resuspended in 500 µL of ice-cold 1x Lysis RSB/NP-40 buffer (10 mM HEPES, pH 7.2, 10 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40), followed by incubation on ice for 10 minutes for complete lysis. The nuclei were sedimented at 1000 g for 2 minutes, and washed with 500 µL RSB/NP-40 buffer. The nuclei were sedimented again at 1000 g for 2 min and resuspended in 1 mL of RSB/NP-40 buffer.

To approximate the concentration of nucleic acids in each sample, 10 µL of the sample was diluted in 990 µL of 0.1% SDS, transferred into a glass cuvette, and exposed to ultraviolet light at a wavelength of 260 nm. The optical density/absorbance at 260 nm wavelength (A₂₆₀) of each sample was measured by a spectrophotometer relative to a SDS blank. The higher the sample concentration, the more ultraviolet light absorbed by

the sample. One A260 unit is known as the concentration of nucleic acids in 1 mL sample to produce an absorbance of 1. One A260 unit of double-stranded DNA (dsDNA) equals 50 µg/mL concentration. The dsDNA concentration in µg/mL was determined by the following equation: [dsDNA] = (A260) (50 µg/mL) (dilution factor (100)). In this study, a total of 10 µg of sample DNA were used for each DNase I digestion experiment.

Each sample was digested with 10 units of RNase-free DNase I (New England Biolabs) (2 U/ml) in a total volume of 1 mL reaction after CaCl₂ (0.5 mM final concentration) was added. 200 µL of reaction mixture was aliquoted at zero time point and the reaction was stopped with an equal volume of stop buffer (5% SDS, 10 mM EDTA, pH 8.0). Four 200 µL aliquots were subsequently stopped in an identical manner at the 5 minute, 10 minute, 20 minute, 30 minute, and 40 minute time points. 25 µL of Proteinase K (400 µg/mL stock) and 4 µL RNase A (10 mg/mL) were added to each aliquot and the aliquots were incubated overnight at 55°C.

Total genomic DNA was extracted from control DNA transfected, plasmid transfected and mock transfected HeLa/406 derivative cells with the E.Z.N.A Tissue DNA kit, as recommended by the manual (Omega Bio-tek). The recovered DNA was resuspended in 50 µL of ddH₂O. 50 ng of genomic DNA was used as a template for each PCR reaction containing Phire Hot Start II DNA Polymerase (Thermo Scientific) and 2 set of primers specific for either the ectopic mutant c-myc (pVU and pDUE) or the β-globin loci (pFor and pRev).

III. RESULTS

Part I: Generation of stable HeLa/FRT.myc.5' (930)-GAL4 mutant cell lines:

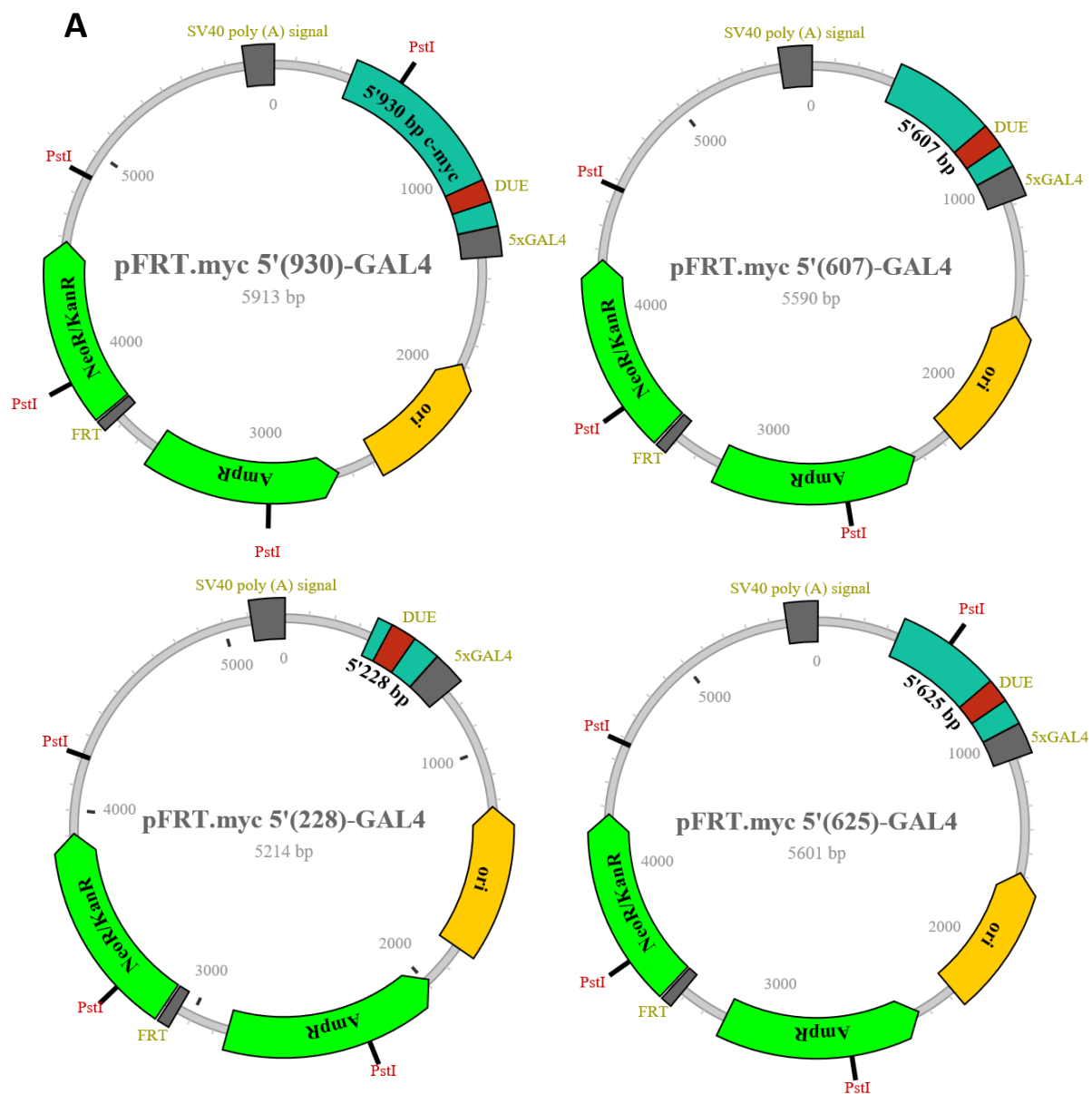
Cloning of deletion constructs of FRT.myc.5' (930)-GAL4:

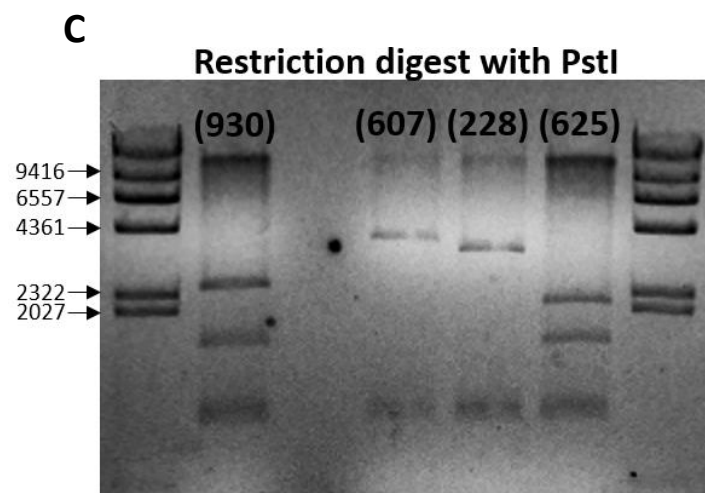
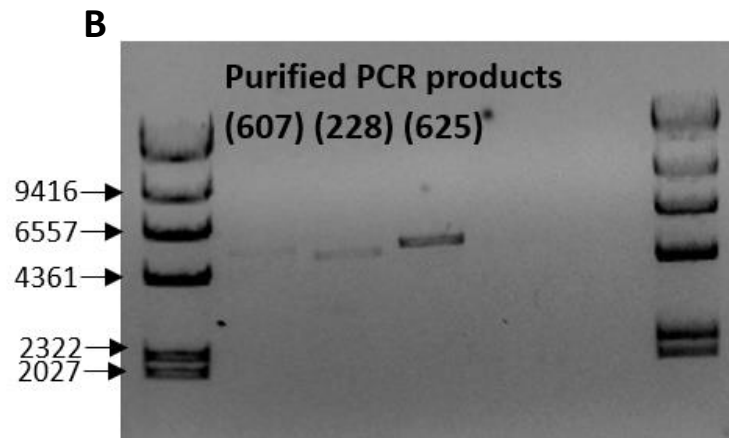
Previous studies suggested that while the deletion of DNA unwinding element (DUE) DNA sequence does not eliminate the pre-replication complex (pre-RC) formation, the DUE is essential for the activation of pre-RC (Ghosh et al., 2006; Chen et al., 2013). Consequently, we are interested in determining the minimal DNA sequence which is required for an active human replication origin. We developed three new deletion mutants of c-myc replicator using the FRT.myc.5'(930)-GAL4 plasmid containing the c-myc replicator, which has a 3' 1420 bp DNA sequence replaced by five tandem GAL-4 binding sites (5xGAL4) (Figure 4, 5a). This plasmid was subjected to inverse PCR using three sets of phosphorylated primers, as depicted in Figure 4 (primer F1-R1 set, primer F2-R2 set, primer F2-R1 set).

The PCR reactions yielded three new c-myc replicator mutants which contain an intact DUE sequence, while lacking 320, 670, 300 bp fragments near the 5' end of c-myc DNA as well as the 3' 1420 bp fragment (Figure 4, 5a). The PCR products were purified and quantified using 1% agarose gel electrophoresis, followed by the ligation reaction using T4 DNase ligase (Figure 5b). The newly circularized PCR products and the original c-myc template (FRT.myc.5'(930)-GAL4) were digested with PstI and separated on 1% agarose gel (Figure 5c). DNA sequencing confirmed the deletions in each construct. The constructs are named based on the remaining length of c-myc DNA in each plasmid, i.e., pFRT.myc.5'(607)-GAL4 has 607 bp of c-myc replicator core. They are listed in the following order: construct (A) is FRT.myc.5'(607)-GAL4 which has bases 381-704 deleted; construct (B) is FRT.myc.5'(625)-GAL4 which has bases 749-1054 deleted; and construct (C) is FRT.myc.5'(228)-GAL4 which has bases 381-1052 deleted.

Figure 5. Construction of truncated FRT.myc.5' (930)-GAL4 plasmids.

- (A) Plasmid maps of pFRT.myc.5'(930)-GAL4, pFRT.myc.5'(607)-GAL4, pFRT.myc.5'(625)-GAL4, and pFRT.myc.5'(353)-GAL4. Black bars: PstI restriction sites. Light green arrows: AmpR (ampicillin-resistant gene), NeoR/KanR (neomycin/kanamycin resistant gene). Gold arrows: Ori (origin of replication). Grey blocks: FRT (FLP-recombinase target site), 5xGAL4 (five tandem GAL4 binding sites), SV40 poly(A) signal. Red block: DUE (DNA unwinding element). Blue green block: c-myc replicator cores with the indicated lengths.
- (B) 1% agarose gel of purified PCR products which were circularized to create (607) (pFRT.myc.5'(607)-GAL4), (228) (pFRT.myc.5'(228)-GAL4), and pFRT.myc.5'(625)-GAL4) constructs.
- (C) EtBr-stained 1% agarose gel of PstI digested plasmids of (930) (pFRT.myc.5'(930)-GAL4) and three new deletion constructs. The expected sizes of digested fragments are listed below the gel image.





Expected sizes (PstI)

(930): 2419, 1579, 992, 923bp
(607): 3645, 992, 923bp
(228): 3296, 992, 923bp
(625): 2077, 1579, 992, 923bp

Making of HeLa/FRT.myc.5' (930)-GAL4 mutant cell lines:

The targeted integration of FRT.myc.5'(607)-GAL4, FRT.myc.5'(228)-GAL4 was accomplished by FLP recombinase-mediated recombination between FRT sites in the c-myc donor plasmid and the chromosomal FRT acceptor site (chromosome 18) in HeLa/406, as previously done with the clonal cell lines containing the full length FRT.myc (Liu et al., 2003) (Figure 6a) or the truncated FRT.myc.5'(930)-GAL4 (Ghosh et al., 2004; Chen et al., 2013). Accurate integration of the new constructs was confirmed by diagnostic PCR primers (mapped out as primers 1, 2, and 3 in the occupied acceptor site, Figure 6a) that yielded products from either the empty or occupied acceptor sites.

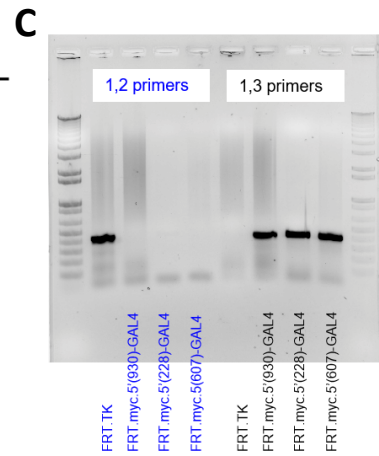
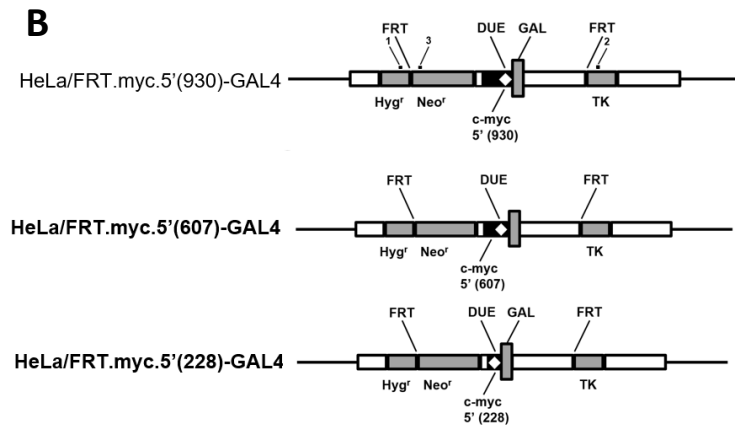
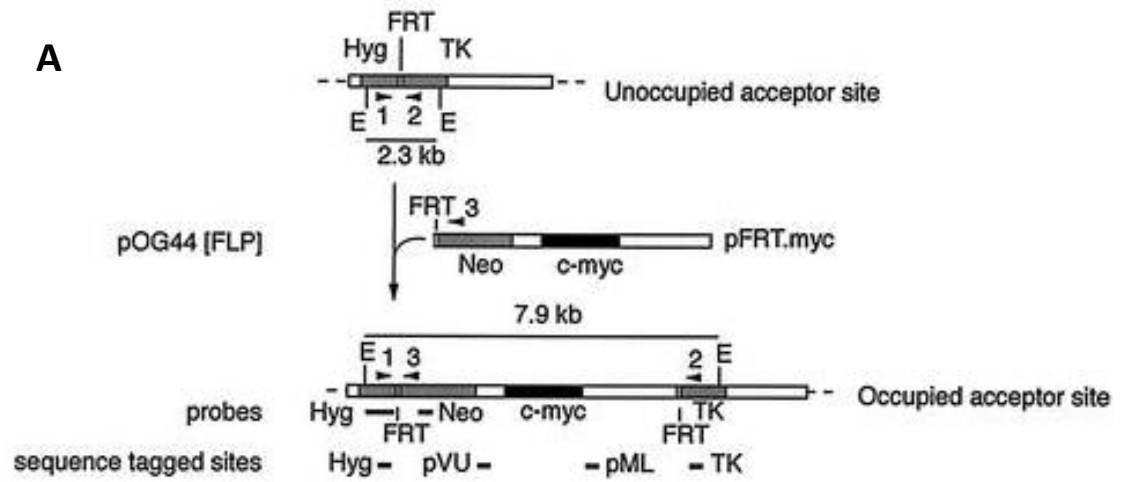
Genomic DNA was isolated from one HeLa/406 empty acceptor cell line (FRT.TK) and three HeLa/406 derivative cell lines containing different c-myc donor plasmids, which are shown in the maps (Figure 6b) as FRT.myc.5' (930)-GAL4, FRT.myc.5' (228)-GAL4, and FRT.myc.5' (607)-GAL4. As expected, diagnostic primers 1 and 2 only amplified a PCR product with HeLa/406(FRT.TK) genomic DNA containing the unoccupied acceptor site, but not with DNA from cells with the occupied acceptor sites by any of the donor plasmids (Figure 6c). On the contrary, primers 1 and 3 amplified products only with DNA from cells with an occupied acceptor site (Figure 6c). These results confirmed the stable integration of the new truncated versions of the c-myc replicator into HeLa/406 acceptor site.

Figure 6. Diagnostic PCR screening of newly constructed ectopic c-myc cell lines.

(A) FLP recombinase-mediated DNA integration into a HeLa ectopic chromosomal site. HeLa/406 cells contain a single chromosomal copy of the plasmid pHyg.FRT.TK, which is known as the unoccupied chromosomal acceptor site. Hyg, hygromycin resistance gene; FRT, FLP recombinase target site; TK, herpes simplex virus thymidine kinase gene. The Hyg-FRT-TK fusion protein renders cells resistant to hygromycin and sensitive to ganciclovir. pFRT.myc (shown linearized) contains the 2.4-kb *HindIII/XhoI* fragment of the *c-myc* origin or its derivatives. Neo, G418 resistance gene with promoter replaced by FRT. pOG44 is an FLP recombinase expression plasmid, cotransfected with pFRT.myc. Accurately targeted cells are resistant to hygromycin, G418, and ganciclovir. PCR primers 1, 2, and 3 (horizontal arrowheads) give products diagnostic for the unoccupied acceptor site (primers 1 and 2) or the occupied acceptor site (primers 1 and 3). Sequence-tagged sites Hyg, pVU, pML, and TK used in Q-PCR quantitation are also indicated (Liu et al., 2003).

(B) Maps of the DNA sequences of the occupied sites in HeLa/406 derivative cell lines. Flanked upstream and downstream by the FRT sites, each donor plasmid contains neomycin gene and one of the three truncated versions (5' 930 bp, 5' 228 bp, and 5' 607 bp) of the *c-myc* replicator (including the DUE) (solid black box) attached to GAL4-binding sites. The bold print indicates the new deletion mutants of *c-myc* replicator.

(C) Genomic DNA from 406-derived cell lines was isolated and amplified by PCR with primer pairs 1 and 2 or 1 and 3. DNA from HeLa/406 empty acceptor cells is shown as FRT.TK. DNA from clonal cell lines HeLa/FRT.myc.5'(930)-GAL4, HeLa/FRT.myc.5'(228)-GAL4, or HeLa/FRT.myc.5'(930)-GAL4 is shown.

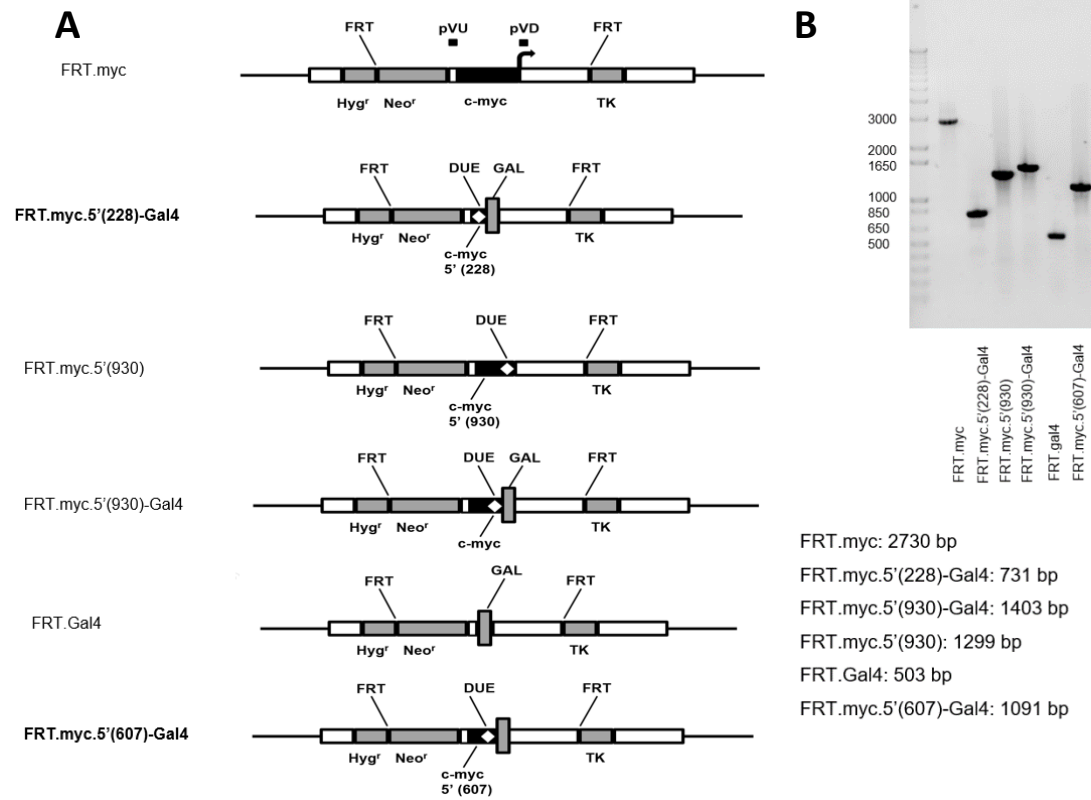


In this study, all clonal cell lines are named for their respective donor plasmids, as listed in Figure 7a. Therefore, at the ectopic chromosomal FRT site, HeLa/FRT.myc cells contain the wild-type 2.4 kb core c-myc replicator; HeLa/FRT.myc. 5'(228)-GAL4 cells contain 5' 228 bp of the c-myc replicator (including the c-myc DUE) flanked by 5xGAL4-binding site cassette; HeLa/FRT.myc.5'(930) cells contain 5' 930 bp of the c-myc replicator without 5xGAL4-binding site cassette; HeLa/FRT.myc. 5'(930)-GAL4 cells contain 5' 930 bp of the c-myc replicator flanked by 5xGAL4-binding site cassette; HeLa/FRT.GAL4 cells contain the 5xGAL4-binding site cassette; HeLa/FRT.myc. 5'(607)-GAL4 cells contain 5' 607 bp of the c-myc replicator flanked by 5xGAL4-binding site cassette. The sizes of ectopic mutant c-myc replicators in these HeLa/406 derivative cells were determined using PCR primers (pVU and pVD) flanking of the c-myc origin, as shown in the FRT.myc in Figure 7a. The PCR results, shown Figure 7b, confirmed that all of HeLa/406 derivative cell lines have the correct donor plasmids.

Figure 7. Size confirmation of the ectopic sites in all HeLa/406 derivative cell lines.

(A) Maps of the ectopic sites in all HeLa/406-derived cell lines. The cell lines are named for their respective pFRT integrants. pVU and pVD are PCR primers flanking the c-myc replicators, as indicated by the small black square.

(B) Genomic DNA from 406-derived cell lines was isolated and amplified by PCR with primer pair pVU and pVD. The expected sizes for the PCR products are listed below the gel image in accordance to their respective lanes on the gel.



Part II: Expression of GAL4^{DBD} fusion proteins:

Cloning and expression of GAL4^{DBD}-wild-type BRG1 and BRG1^{K798R} mutant:

Plasmids pEGFP GAL4^{DBD} - wild-type BRG1 and BRG1^{K798R} mutant were digested with PstI and NheI (Figure 8a) in order to extract the cDNAs of GAL4^{DBD} fused with wild-type BRG1 or BRG1^{K798R} mutant. The vector pZFN-BSD (blasticidin S deaminase) contained the zinc finger (ZFN) cDNA, which was removed by NheI and PmeI, generating compatible ends as the inserts (GAL4-wild-type BRG1 or BRG1^{K798R} mutant) (Figure 8b). Both the linearized pZFN-BSD vector and the insert fragments of GAL4^{DBD}-wild-type BRG1 and BRG1^{K798R} mutant were gel extracted from 1% agarose gel before purification by E.Z.N.A Gel Extraction kit. The ligated products GAL4^{DBD}-wild-type BRG1-BSD and GAL4^{DBD}-BRG1^{K798R}-BSD were confirmed after two diagnostic restriction digests, the first one with BamHI, and the second one with SacII and SpeI. The sizes of digested fragments were predicted via Snapgene construct map of GAL4^{DBD}-BRG1-BSD.

The correct GAL4^{DBD}-wild-type BRG1-BSD and GAL4^{DBD}-BRG1^{K798R}-BSD plasmids with appropriately cut DNA fragments were shown in Figure 9b. The sequences of these plasmids were further verified by DNA sequencing. Consequently, the plasmids expressing GAL4^{DBD} fusions with wild-type BRG1 or the catalytically inactive BRG1^{K798R} mutant were transiently transfected in HeLa cells for 24 hours, followed by 24 hours of blasticidin treatment. Because these plasmids contain the blasticidin S deaminase gene, which codes for the blasticidin-degrading enzyme, the positively-transfected cells are rendered blasticidin resistant. As shown in Figure 9c, GAL4^{DBD}-wild-type BRG1 treated cells show modest expression while GAL4^{DBD}-BRG1^{K798R} mutant shows twice as much expression (the asterisk indicates the GAL4-specific band; beta-actin was used as the loading control). The vector pZFN-BSD treated cells, as expected, show no expression of GAL4^{DBD}-BRG1 against anti-GAL4^{DBD} antibody.

Figure 8. Cloning of GAL4^{DBD}-wild-type BRG1-BSD and GAL4^{DBD}-BRG1^{K798R}-BSD.

(A) Vector preparation. Left: Plasmid map of vector pZFN-BSD (blasticidin S deaminase). The light purple region indicated the cDNA of zinc-finger nuclease (ZFN) which would be replaced by the cDNA of GAL4^{DBD}-wild-type BRG1. The restriction sites PmeI and NheI (red) were used to linearize the vector. Right: The vector pZFN-BSD was digested by PmeI and NheI restriction enzymes in order to remove the ZFN cDNA. The right fragment (4956 bp) was gel extracted and purified before ligation. (M) indicates Gene Ruler 1 kb plus DNA ladder (Thermo Scientific). The expected sizes of vector fragments are shown below the gel image.

(B) Insert preparation. Left: Plasmid map of insert pEGFP GAL4^{DBD}-wild-type BRG1. Since the -BRG1^{K798R} mutant is caused by a missense point mutation, the length of the DNA sequenced and the protein molecular weight are unchanged. The purple region indicated the cDNA of GAL4^{DBD}-wild-type BRG1, which will be used as the insert during ligation. The restriction sites NheI and PsiI (red) were used to extract the insert. Right: The inserts pEGFP GAL4^{DBD}-wild-type BRG1 or pEGFP GAL4^{DBD}-BRG1^{K798R} mutant were digested with NheI and PsiI, and separated by agarose gel electrophoresis. The right fragment (5373 bp) was gel extracted and purified before ligation. WT indicates wild-type BRG1 and MT indicates BRG1^{K798R} mutant. The expected sizes of digested pEGFP GAL4^{DBD}-wild-type BRG1 after NheI and PsiI digestion are shown.

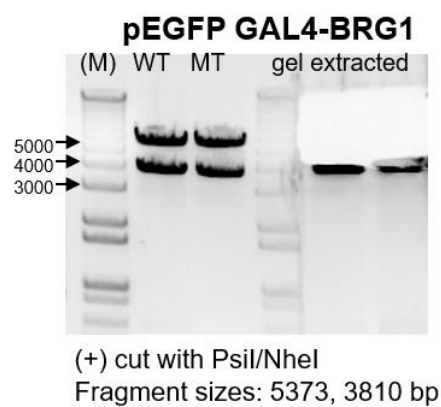
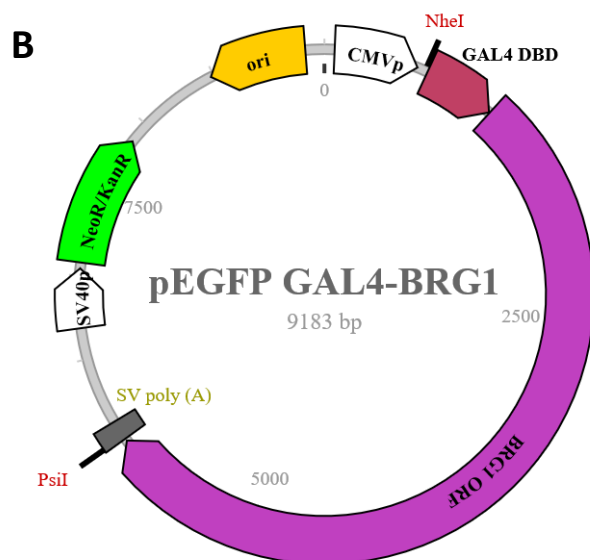
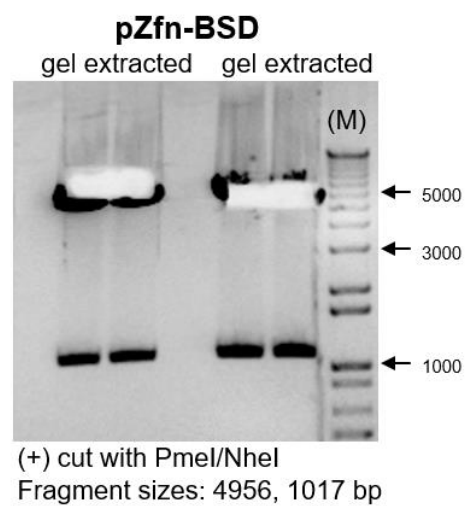
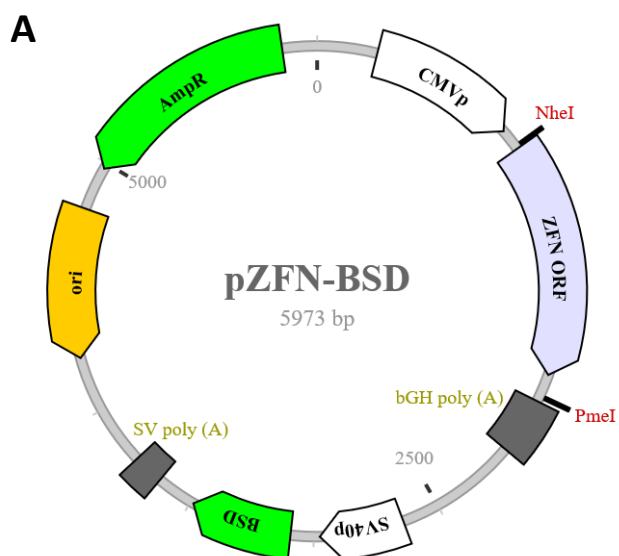
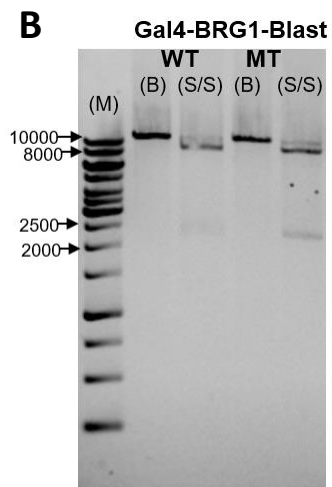
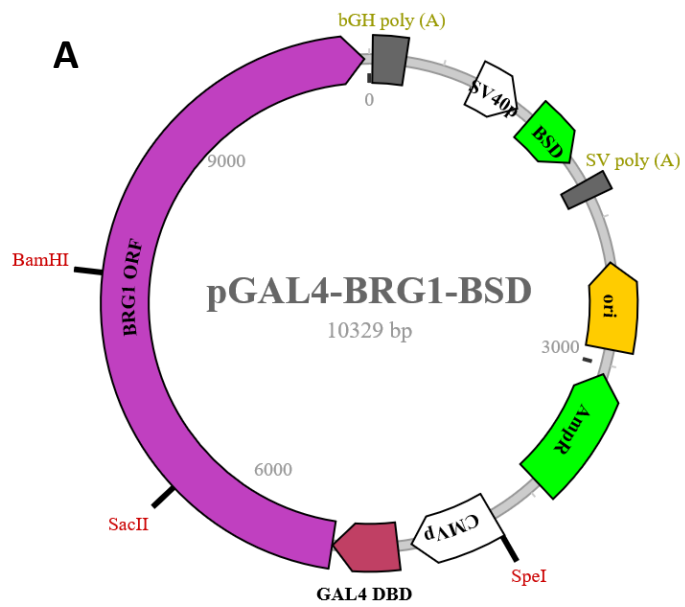


Figure 9. Confirmation of GAL4^{DBD}-wild-type BRG1-BSD and GAL4^{DBD}-BRG1^{K798R}-BSD.

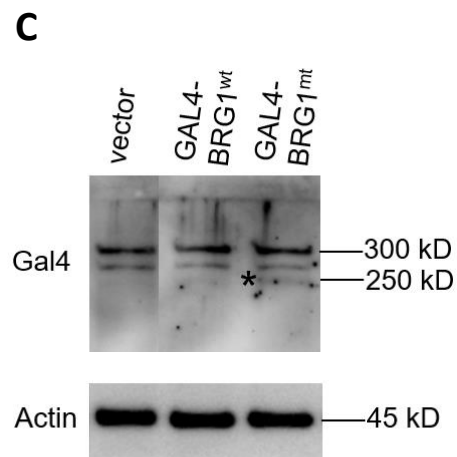
(A) Plasmid map of plasmid GAL4^{DBD}-wild-type BRG1-BSD. GAL4^{DBD}-wild-type BRG1 and BSD cDNAs are in purple and green, respectively. CMVp in white denotes the DNA sequences of CMV promoter and enhancer. SV40p in white denotes the DNA sequences of SV40 promoter and SV40 origin of replication. BamHI, SacII, and SpeI restriction sites are in red.

(B) The correct plasmid DNAs of GAL4^{DBD}-wild-type BRG1-BSD and GAL4^{DBD}-BRG1^{K798R}-BSD were linearized by BamHI or digested by SacII and SpeI. (M) indicates Gene Ruler 1 kb DNA ladder (Thermo Scientific). (B) indicates BamHI-linearized plasmid DNA, (S/S) indicates SacII and SpeI DNA fragments. The expected sizes of DNA fragments are shown.

(C) Western blot with anti-GAL4 antibody of extracts from HeLa cells transfected with vector only (lane 1), cells expressing GAL4^{DBD}-wild-type BRG1 (lane 2), and cells expressing GAL4^{DBD}-BRG1^{K798R} mutant (lane 3). The asterisk indicates the GAL4^{DBD} fusion protein of wild-type and mutant form of BRG1. Beta-actin protein expression was used as a loading control.



(B) BamHI cut fragment: 10357 bp
 (S/S) SacII/SpeI cut fragments: 8137, 2220 bp



Part III: Chromatin structures of the ectopic mutant c-myc replicators in HeLa cells:

Previous study (Chen et al., 2013) has shown that several replication proteins (HBO1, Cdt1, Orc2) can be recruited to the inactivated 5' 930 bp c-myc replicator using GAL4 protein- GAL4 DNA attachment, leading to the reactivation of the truncated replicator and the recruitment of other endogenous replication proteins (Mcm2-7, ORC, Cdc45, and DUE-B). In this study, the chromatin structure of the inactivated 5' 930 bp c-myc replicator in the presence of GAL4^{DBD} fusion protein (Cdt1 and BRG1) tethering was further elucidated using DNase I hypersensitivity assay. Cdt1 is a good candidate protein because it has been shown to restore origin activity when being fused with GAL4^{DBD} protein. BRG1 (SMARCA4) is the catalytic component of the human SWI/SNF chromatin remodeling complex. Wild-type BRG1 has been shown to use ATPase activity to disrupt histone-DNA contacts, leading to the repositioning of the nucleosome and exposure of naked DNA for transcription (Wilson and Roberts, 2011). Thus, we hypothesize that this protein, once fused with GAL4^{DBD} protein, increases the nuclease sensitivity of inactivated FRT.myc.5'(930)-GAL4 origin.

DNase I digestion and nuclease sensitivity of HeLa/FRT.myc.5'(930)-GAL4 cells:

Before DNase I hypersensitivity assay (previously described in Methods section, Figure 10) was performed on GAL4^{DBD} fusion protein-transfected HeLa cells, DNase digestion and PCR across the ectopic c-myc and beta-globin loci were used to evaluate chromatin structures of non-transfected HeLa/FRT.myc.5'(930)-GAL4 cells. Time-dependent DNase-digested HeLa/FRT.myc.5'(930)-GAL4 genomic DNA samples from two separate experiments were visualized on EtBr-stained 1% agarose gel, as shown in Figure 11b. The DNA smears were formed due to a series of fragmented DNA in various sizes. The longer the nuclei were treated with DNase I, the longer the smears appear on the gel, which indicated that nucleosomal DNA were being digested by DNase I in a time-dependent manner.

The same genomic DNA samples extracted from DNase-digested HeLa/FRT.myc.5'(930)-GAL4 nuclei were amplified simultaneously by PCR primers (primers pVU, pDUE, pFor, pRev) that yielded products from both the ectopic c-myc and β -globin loci. As seen in Figure 11c, primers pVU and pDUE amplified a PCR product

1157 bp while β -globin primers pFor and pRev amplified a 668 bp PCR product. The intensity of β -globin product remains unchanged across time points, which indicates that β -globin chromatin is in a closed confirmation and inaccessible to DNaseI. This data is consistent with previous study that showed modest β -globin origin activity, indicated by the low nascent-strand abundance of several initiation sites within the 40 kb region of the β -globin gene locus (Kamath and Leffak, 2001). Therefore, β -globin locus was used as an internal normalizer for DNase I sensitivity.

Subsequently, the DNase I sensitivity of the ectopic c-myc replicator was determined by the ratio of the band intensity of PCR product specific for the β -globin locus over that of the ectopic c-myc locus (Ratio = β -globin/ectopic). Thus, the higher this ratio, the greater the sensitivity of the ectopic site relative to the β -globin locus. Non-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were shown to have a slight nuclease sensitivity at the ectopic c-myc locus, indicated by a decreasing trend in band intensity of ectopic PCR products at later time points (20, 30, and 40 minutes) (Figure 11c). This result can be explained by the various DNA structures within the 5' 930 bp of the c-myc replicator, such as the easily unwound AT rich DUE sequence. However, the 5' 930 bp of the c-myc replicator has been shown to remain inactive in the absence of GAL4^{DBD} fusion protein tethering (Chen et al., 2013). Thus, the c-myc core replicator is a nuclease-accessible ('open') chromatin structure.

Figure 10. Schematic experimental design of DNase I hypersensitivity assay.

The HeLa/406-derived cells were transiently transfected with GAL4^{DBD} fusion proteins of interest (Cdt1, wild-type BRG1 or BRG1^{K798R} mutant) for 24 hours to 48 hours. The cells were harvested, followed by lysis using RSB/NP-40 buffer and resuspension of isolated nuclei. Subsequently, the nuclear DNA was digested by DNase I in a time-dependent manner. After the digestion, the genomic DNA was extracted, purified, and amplified simultaneously via PCR using primer sets specific for the ectopic c-myc (pVU and pDUE) and β -globin (pFor, pRev) loci.

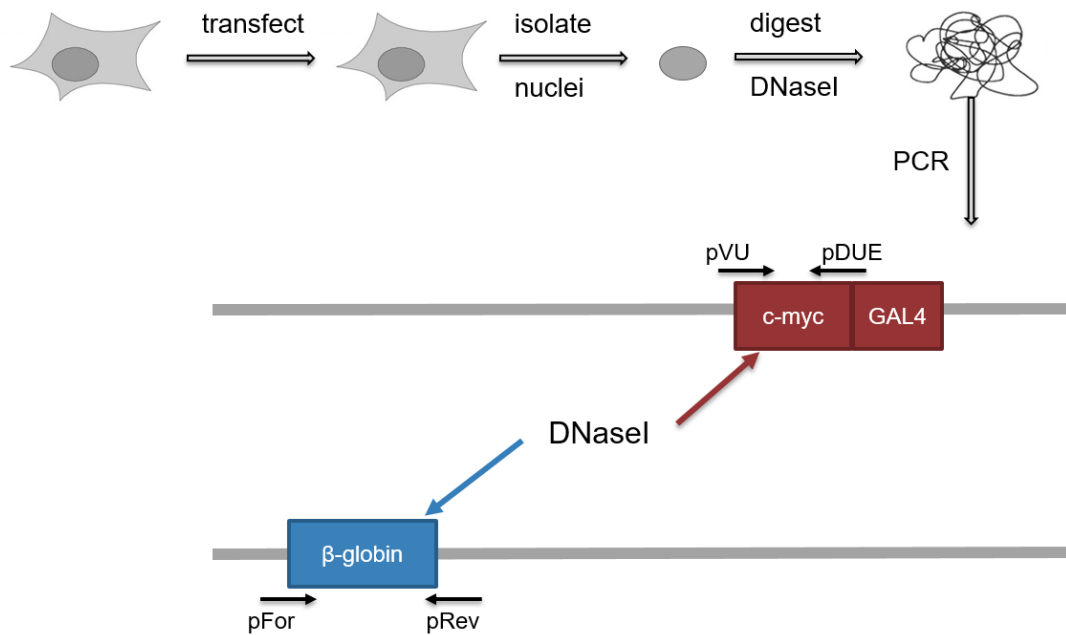
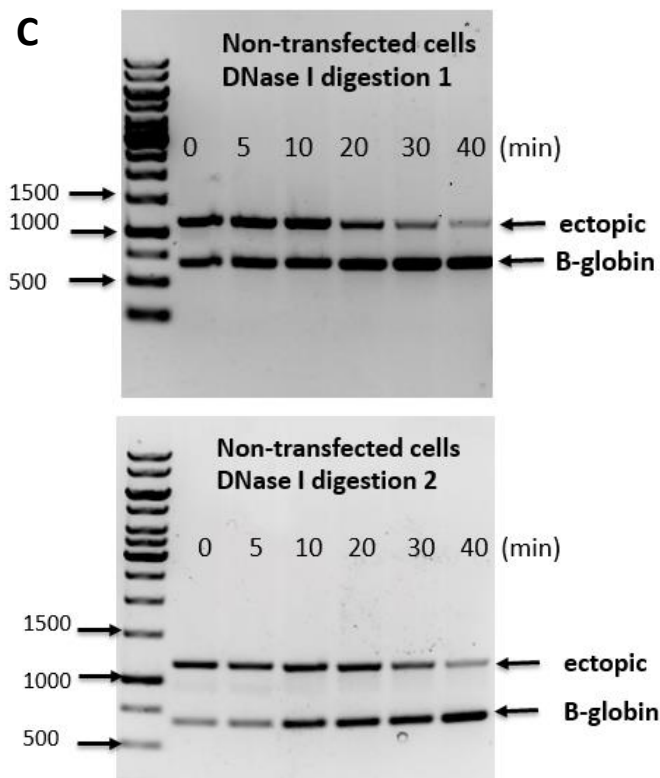
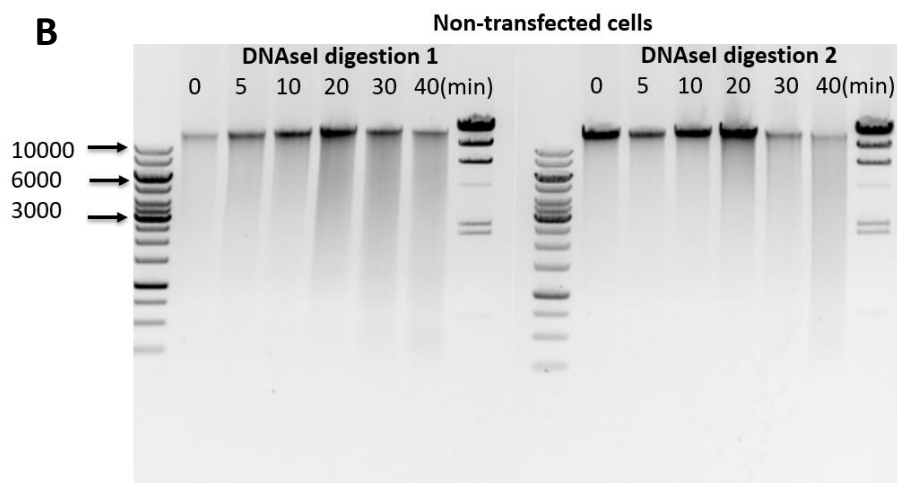
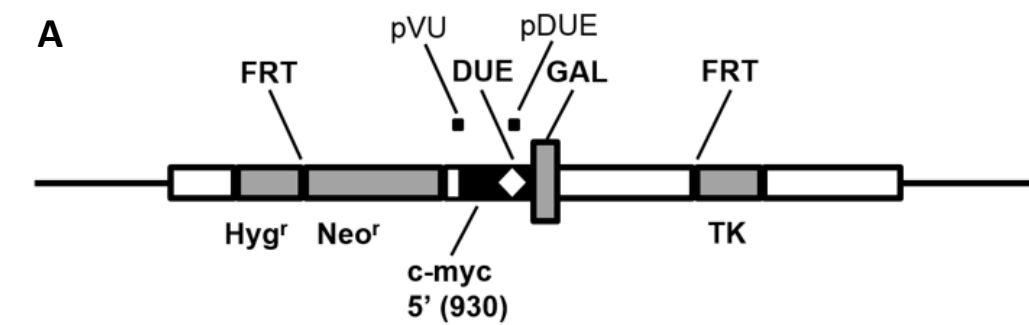


Figure 11. Nuclease sensitivity of non-transfected HeLa/FRT.myc.5'(930)-GAL4 cells. (A) Map of the ectopic c-myc locus in HeLa/FRT.myc.5'(930)-GAL4 cells. The solid box shows the 5' 930 bp of the inactivated c-myc replicator. The small black squares show the positions of pVU and pDUE primers on the c-myc replicator.

(B) Genomic DNAs of time-dependent DNase I-treated non-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were extracted and purified, followed by visualization via agarose gel electrophoresis. The two agarose gel images were results from two separate digestions.

(C) The same genomic DNAs were used as templates for PCR with primers which amplified across both the ectopic c-myc and β -globin loci. The PCR products were visualized on 1% agarose gel and labelled according to the locus from which they were amplified.



Expression of GAL4^{DBD}-Cdt1 increases the nuclease sensitivity only in HeLa/FRT.myc.5'(930)-GAL4 cells, and not HeLa/FRT.myc.5'(930) cells:

GAL4^{DBD}-Cdt1 or GFP proteins were expressed in the FRT cell lines and visualized by anti-Cdt1 antibody (Figure 12a). In comparison to GFP-transfected samples, expression of GAL4^{DBD}-Cdt1 induced a two-fold increase in nuclease sensitivity of the ectopic c-myc origin in HeLa/FRT.myc.5'(930)-GAL4 cells (Figure 12b). The DNase I hypersensitivity of non-transfected, GFP-N1 vector-transfected, GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were quantified using linear regression of the band intensity ratios of ectopic c-myc PCR product over β -globin PCR product (β -globin/ectopic ratio) (Figure 12c). Each treatment was done in triplicate; the slope of each linear regression indicated the increasing rate of β -globin/ectopic ratios; and the Y intercept was set at one for all linear regression trend lines.

In contrast, expression of GAL4^{DBD}-Cdt1 did not induce an increase in nuclease sensitivity of the ectopic c-myc origin in HeLa/FRT.myc.5'(930) cells which do not contain a GAL4 binding site (Figure 13b). The β -globin/ectopic intensity ratios were approximately one across time points in both mock and GAL4^{DBD}-Cdt1-transfected samples, as shown in the scatter plot (Figure 13c). These results were consistent with previous study which showed that expression of GAL4^{DBD}-Cdt1 to the inactivated FRT.myc.5'(930)-GAL4 replicator restored origin activity, due to the tethering between GAL4^{DBD} fused protein and GAL4 DNA sequence. However, without a GAL4 binding site, no tethering was achieved with the inactivated FRT.myc.5'(930) replicator, which led to no restored origin activity. These results also confirmed the hypothesis that the chromatin structure of the FRT.myc.5'(930)-GAL4 becomes more accessible to nuclease in the presence of replication proteins (Cdt1 in this experiment) at the ectopic c-myc replicator.

Figure 12. GAL4^{DBD}-Cdt1 fusion protein increased the nuclease sensitivity of the ectopic c-myc origin in HeLa/FRT.myc.5'(930)-GAL4 cells.

(A) Western blot with anti-Cdt1 antibody of extracts from HeLa/FRT.myc.5'(930)-GAL4 cells expressing GAL4^{DBD}-Cdt1 (left lane), and cells transfected with vector only (right lane). The arrows represent protein bands of endogenous Cdt1 and GAL4^{DBD}-Cdt1.

(B) Genomic DNAs of time-dependent DNase I-treated of GFP-N1 vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were extracted and purified before PCR amplification. The PCR products specific to the ectopic c-myc locus (1157 bp) or β -globin locus (668 bp) were visualized on agarose DNA gel.

(C) The ratios of band intensities of ectopic c-myc PCR product over β -globin product (β -globin/ectopic ratios) from non-transfected, vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were scatter-plotted and fitted for linear regressions. Each treatment was repeated three times, and the DNase I hypersensitivity of each treatment is quantified by the slope of each linear regression. Red squares indicate GFP-N1 vector-transfected DNA samples, and blue diamonds indicate GAL4^{DBD}-Cdt1-transfected DNA samples, and green triangles indicate non-transfected DNA samples.

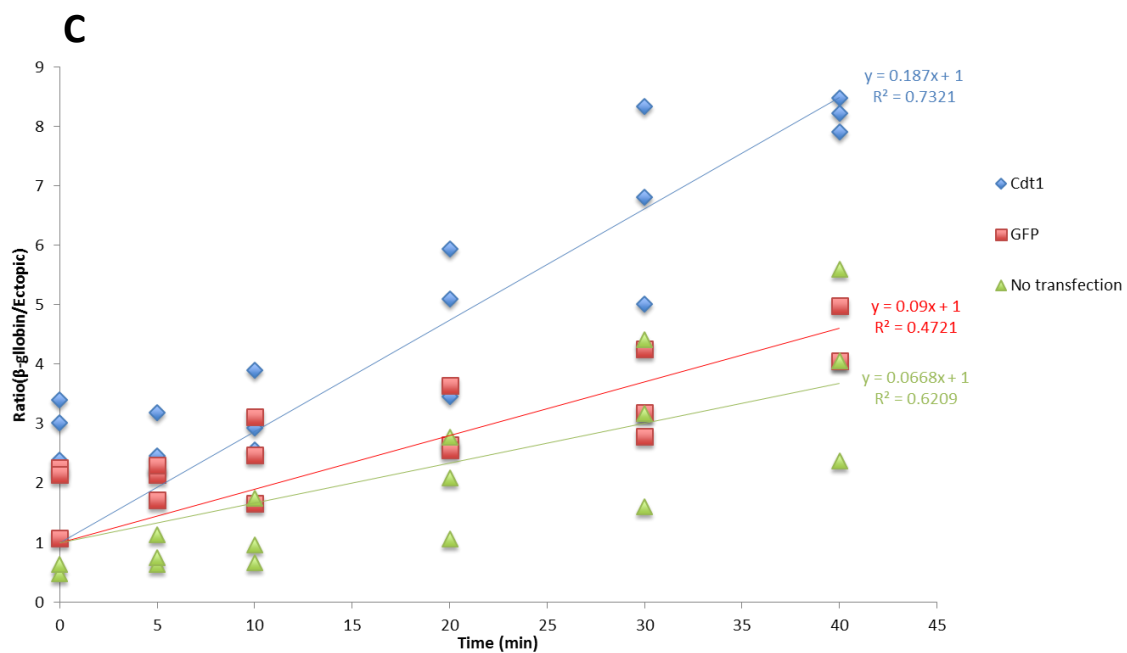
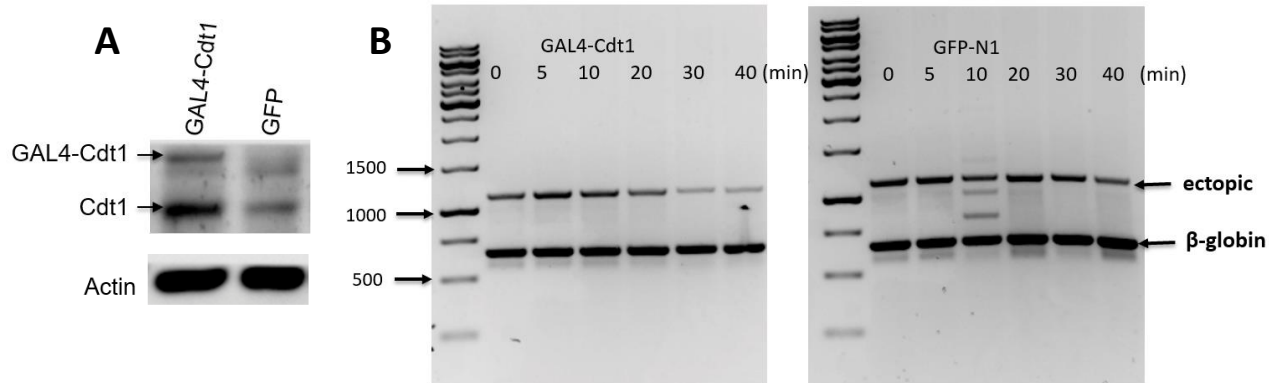
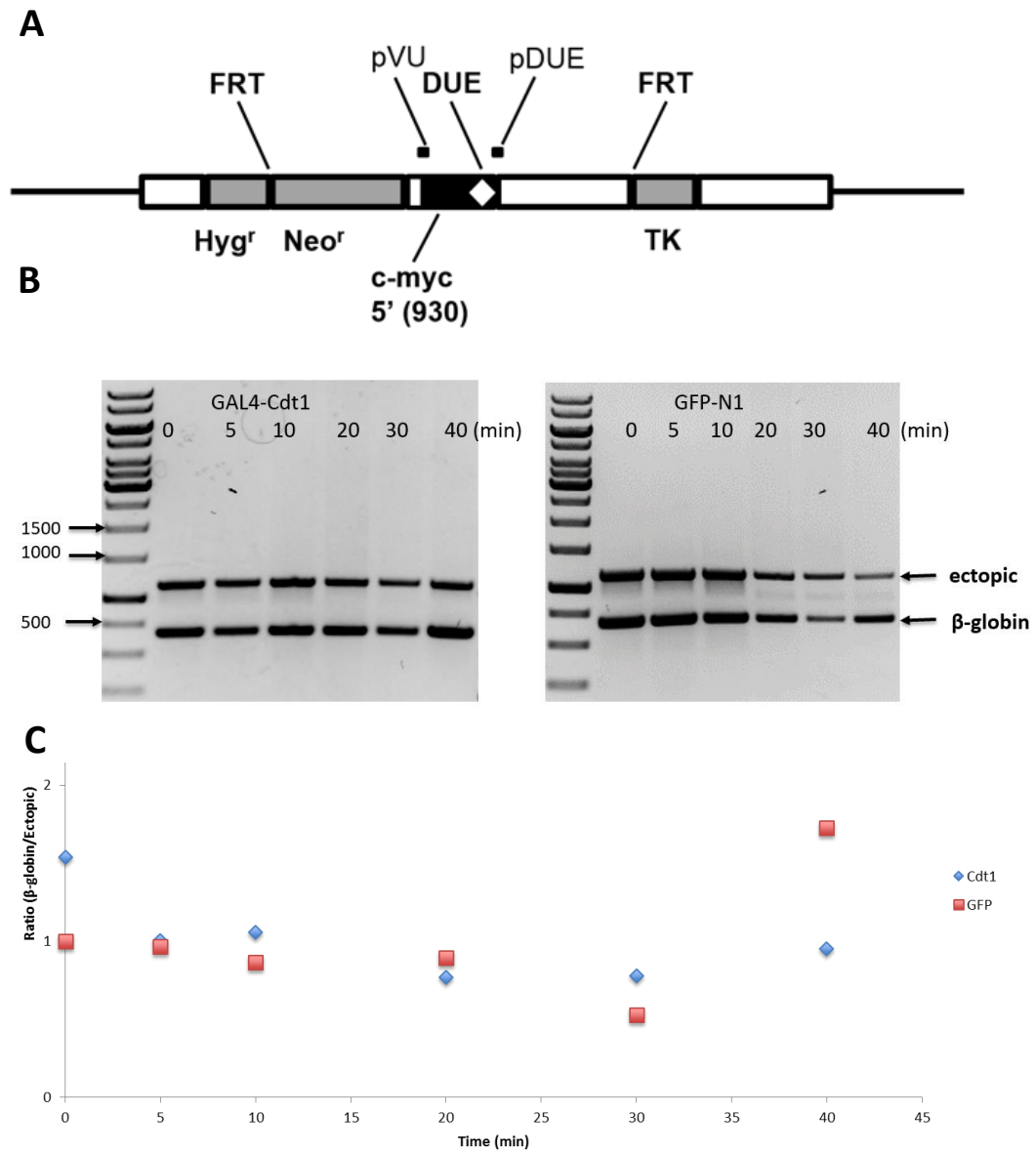


Figure 13. GAL4^{DBD}-Cdt1 fusion protein did not change the nuclease sensitivity of the c-ectopic c-myc origin in HeLa/FRT.myc.5'(930) cells.

(A) Map of the ectopic c-myc locus in HeLa/FRT.myc.5'(930) cells.

(B) Genomic DNAs of time-dependent DNase I -treated of GFP-N1 vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(930) cells were extracted and purified before PCR amplification. The PCR products specific to the ectopic c-myc (1157 bp) or β -globin locus (668 bp) were visualized on agarose DNA gel.

(C) The β -globin/ectopic ratios of vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(930) cells were plotted. Red squares indicate GFP-N1 vector-transfected DNA samples, and blue diamonds indicate GAL4^{DBD}-Cdt1-transfected DNA samples.



Expression of GAL4^{DBD}-Cdt1 did not increase the nuclease sensitivity in HeLa/FRT.myc.5'(607)-GAL4 cells, or HeLa/FRT.myc.5'(228)-GAL4 cells:

As previously mentioned, two more HeLa/FRT cell lines were created with 5' 607 bp and 5' 228 bp c-myc replicators flanked by 5xGAL4 sequence. We were interested in the nuclease sensitivity of these cell lines because while they retained the DUE sequence, they had additional deletions of 320 bp or 600 bp upstream of the DUE. The HeLa/FRT.myc.5'(607)-GAL4 and HeLa/FRT.myc.5'(228)-GAL4 cells were transfected with GAL4^{DBD}-Cdt1 and digested in the same procedure as that of HeLa/FRT.myc.5'(930)-GAL4 cells. GAL4^{DBD}-Cdt1 expression in these cell lines was visualized by anti-Cdt1 in conjunction with endogenous Cdt1 (Figures 14a, 15a). The GAL4^{DBD}-Cdt1 protein expression in HeLa/FRT.myc.5'(930)-GAL4 was comparable to that in HeLa/FRT.myc.5'(607)-GAL4, and approximately four times more than that in HeLa/FRT.myc.5'(607)-GAL4 (Figures 12a, 14a, 15a). However, because GAL4^{DBD}-Cdt1 was in great excess over GAL4 binding sites, we expected that lower concentration of GAL4^{DBD}-Cdt1 does not affect the occupancy of GAL4^{DBD}-Cdt1 at the ectopic sites.

In comparison to mock GFP-transfected samples, GAL4^{DBD}-Cdt1-transfected samples had no significant change in nuclease sensitivity in both FRT.myc.5'(607)-GAL4 and FRT.myc.5'(228)-GAL4 replicators (Figures 14c, 15c). Furthermore, the scatter plots for both HeLa/FRT.myc.5'(607)-GAL4 and HeLa/FRT.myc.5'(228)-GAL4 also confirmed that the β -globin/ectopic ratios were not different between GFP-transfected GAL4^{DBD}-Cdt1-transfected samples (Figures 14d, 15d).

These results indicated that the chromosomal DNA of the ectopic mutant c-myc replicators (FRT.myc.5'(607)-GAL4 and FRT.myc.5'(228)-GAL4) remained in a condensed state and hence became inaccessible to DNase I nuclease. Consequently, these results suggest that GAL4^{DBD}-Cdt1 tethering to the c-myc DUE sequence might not be sufficient, and that additional DNA sequences are required in conjunction with the DUE to unwind the origin DNA necessary for the restored origin activity of these truncated c-myc replicators.

Figure 14. GAL4^{DBD}-Cdt1 fusion protein did not change the nuclease sensitivity of HeLa/FRT.myc.5'(607)-GAL4 cells.

(A) Western blot with anti-Cdt1 antibody of extracts from HeLa/FRT.myc.5'(607)-GAL4 cells expressing GAL4^{DBD}-Cdt1 (left lane), and cells transfected with vector only (right lane). The arrows represent protein bands of endogenous Cdt1 and GAL4^{DBD}-Cdt1.

(B) Map of the ectopic c-myc locus in HeLa/FRT.myc.5'(607)-GAL4 cells.

(C) Genomic DNAs of time-dependent DNase I-treated of GFP-N1 vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(607)-GAL4 cells were extracted and purified before PCR amplification. The PCR products specific to the ectopic c-myc locus (834 bp) or β -globin locus (668 bp) were visualized on agarose DNA gel.

(D) The β -globin/ectopic ratios of vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(607)-GAL4 cells were plotted. Red squares indicate GFP-N1 vector-transfected DNA samples, and blue diamonds indicate GAL4^{DBD}-Cdt1-transfected DNA samples.

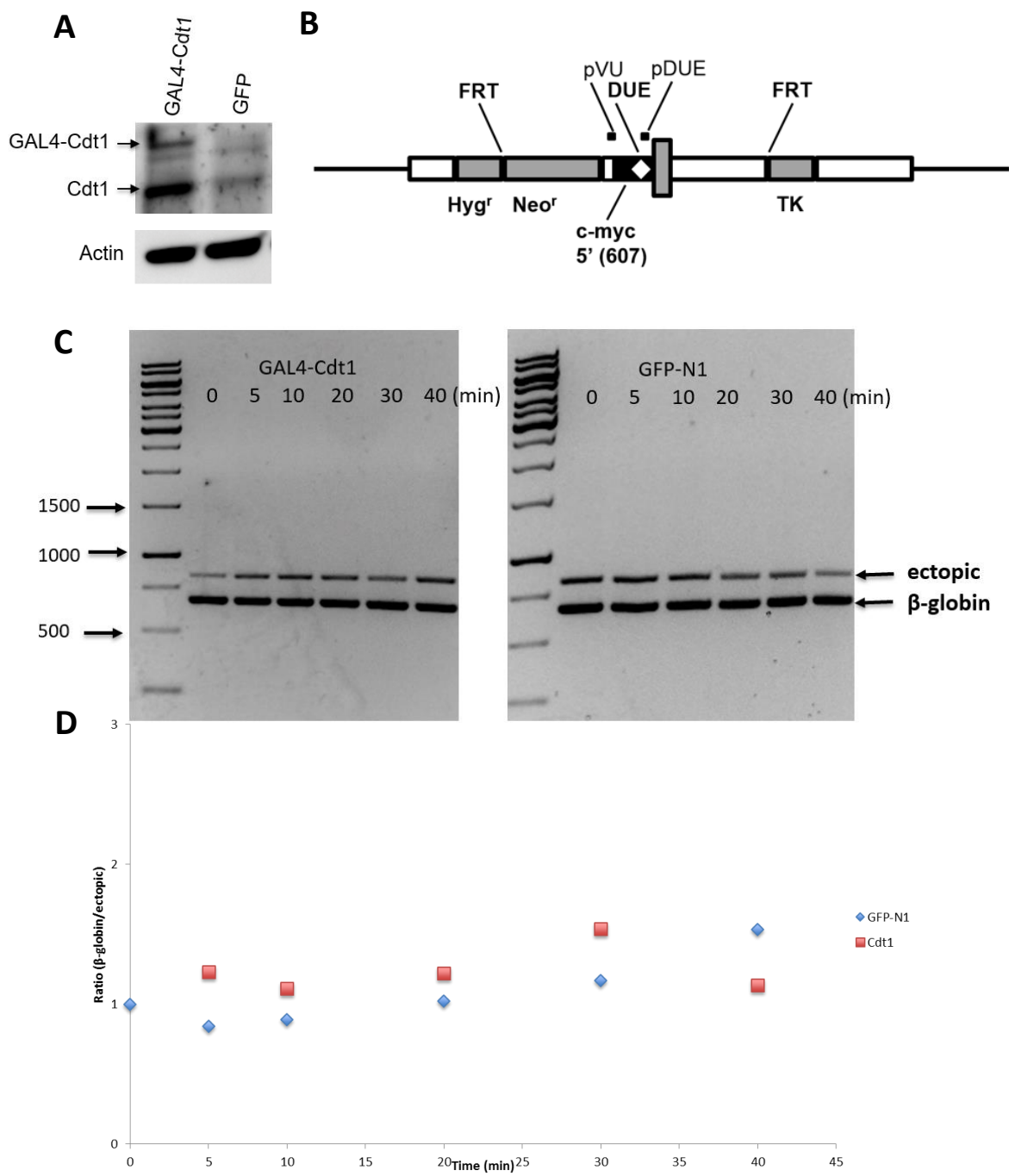
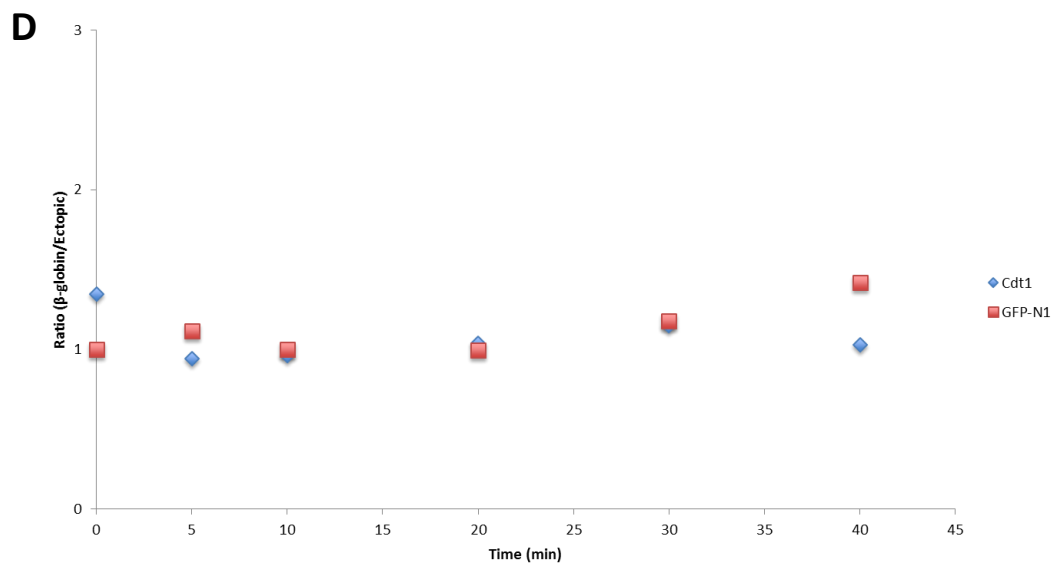
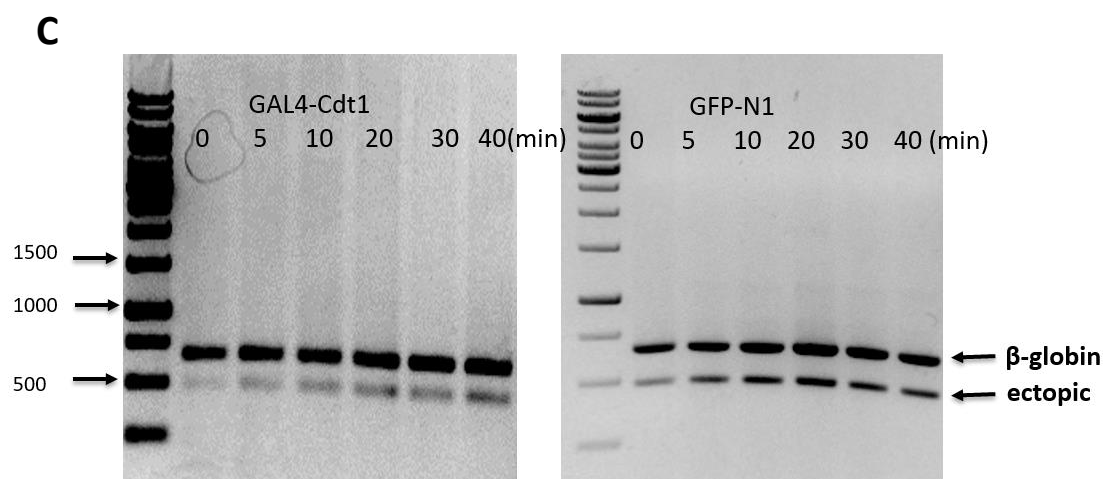
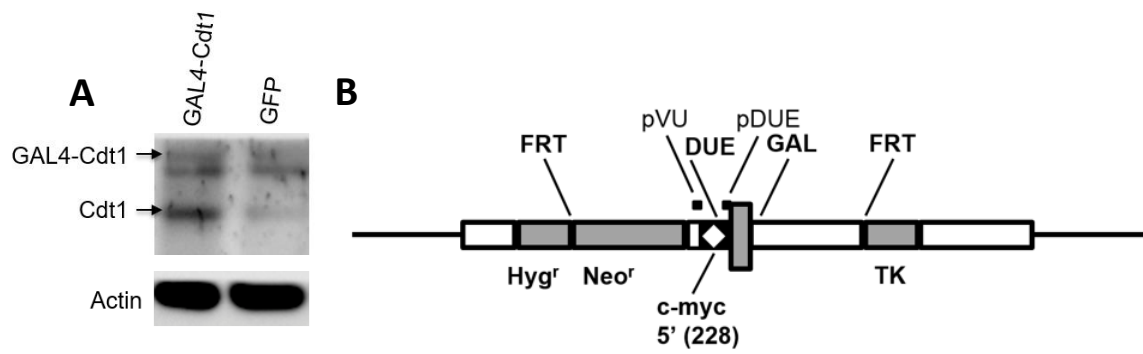


Figure 15. GAL4^{DBD}-Cdt1 fusion protein did not change the nuclease sensitivity of HeLa/FRT.myc.5'(228)-GAL4 cells.

(A) Western blot with anti-Cdt1 antibody of extracts from HeLa/FRT.myc.5'(228)-GAL4 cells expressing GAL4^{DBD}-Cdt1 (left lane), and cells transfected with vector only (right lane). The arrows represent protein bands of endogenous Cdt1 and GAL4^{DBD}-Cdt1.

(B) Map of the ectopic c-myc locus in HeLa/FRT.myc.5'(228)-GAL4 cells.

(C) Genomic DNAs of time-dependent DNase I-treated of GFP-N1 vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(228)-GAL4 cells were extracted and purified before PCR amplification. The PCR products of specific to the ectopic c-myc locus (455 bp) or β -globin locus (668 bp) were visualized on agarose DNA gel. (C) The β -globin/ectopic ratios of vector-transfected, and GAL4^{DBD}-Cdt1-transfected HeLa/FRT.myc.5'(228)-GAL4 cells were plotted. Red squares indicate GFP-N1 vector-transfected DNA samples, and blue diamonds indicate GAL4^{DBD}-Cdt1-transfected DNA samples.



Expression of GAL4^{DBD}-wild-type BRG1 or BRG1^{K798R} fusion proteins increases the nuclease sensitivity in HeLa/FRT.myc.5'(930)-GAL4 cells:

GAL4^{DBD}-wild-type BRG1, GAL4^{DBD}-BRG1^{K798R} mutant, and pZFN-BSD vector were expressed in HeLa/FRT.myc.5'(930)-GAL4 cells and visualized by anti-GAL4 antibody (Figure 17a). After normalization by beta-actin loading control, the expression of GAL4^{DBD}-BRG1^{K798R} was shown to be two times stronger than that of GAL4^{DBD}-wild-type BRG1. This result was consistent with previous expression profile of GAL4^{DBD}-wild-type BRG1, GAL4^{DBD}-BRG1^{K798R} mutant (Figure 9c).

In order to confirm that the DNase I was the only nuclease affecting the sensitivity of HeLa/FRT nucleosomal DNA, the nuclei samples from vector-transfected cells were treated identically with or without DNase I addition. As a result, the non-treated nuclei showed no nuclease sensitivity at both ectopic c-myc and β -globin loci, up to 40 minutes (Figure 16a), whereas the DNase I-treated nuclei showed moderate sensitivity at the ectopic c-myc locus (Figure 16b). This experiment confirmed that endogenous nucleases did not interfere with the DNase I hypersensitivity assay and DNase I was the main enzyme digesting nucleosomal DNA in these HeLa/FRT cell lines.

Subsequently, PCR products of HeLa/FRT.myc.5'(930)-GAL4 expressing either GAL4^{DBD}-wild-type BRG1 or BRG1^{K798R} mutant were visualized on 1% agarose gel (Figure 17b). In comparison to empty vector-transfected samples (Figure 16b), GAL4^{DBD} wild-type BRG1-transfected samples (both wild-type and mutant forms) had increased nuclease sensitivities at the ectopic c-myc locus, indicated by a faster decreasing trend in band intensities of ectopic PCR products. Furthermore, the scatter plot of each treatment (Figure 17c) confirmed the increased DNase I sensitivity of FRT.myc.5'(930)-GAL4 replicator when expressed GAL4^{DBD}- wild-type BRG1 or BRG1^{K798R} mutant. Using the slope of each linear regression to approximate the increasing rate of the β -globin/ectopic ratio, the DNase I sensitivity of wild-type BRG1-transfected and BRG1^{K798R} mutant-transfected samples was respectively 3.6 times and 2.6 times higher than that of vector-transfected samples.

These results supported the hypothesis that GAL4^{DBD}- wild-type BRG1 fusion protein might be able to modify chromatin structure of the inactivated FRT.myc.5'(930)-GAL4 replicator, leading to increased nuclease sensitivity. The catalytic-dead BRG1^{K798R}

mutant, while it still sensitized the ectopic c-myc origin to DNase I, decreased the DNase I sensitivity of HeLa/FRT.myc.5'(930)-GAL4 by 27%. As shown in Figure 17a, the expression profile of GAL4^{DBD}- wild-type BRG1 in the cells was only half of that GAL4^{DBD}- BRG1^{K798R} mutant. Thus, if GAL4^{DBD}- wild-type BRG1 was expressed to the same level as that of GAL4^{DBD}- BRG1^{K798R}, the fusion protein might have further sensitized the inactivated c-myc origin.

Figure 16. Endogenous nucleases did not interfere with DNase I hypersensitivity assay.

(A) Genomic DNAs of time-dependent non-treated vector-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were extracted and purified before PCR amplification. The PCR products specific to the ectopic c-myc locus (1157 bp) or β -globin locus (668 bp) were visualized on agarose DNA gel.

(B) Genomic DNAs of time-dependent DNase I-treated vector-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were extracted and purified before PCR amplification. The PCR products were visualized on agarose DNA gel.

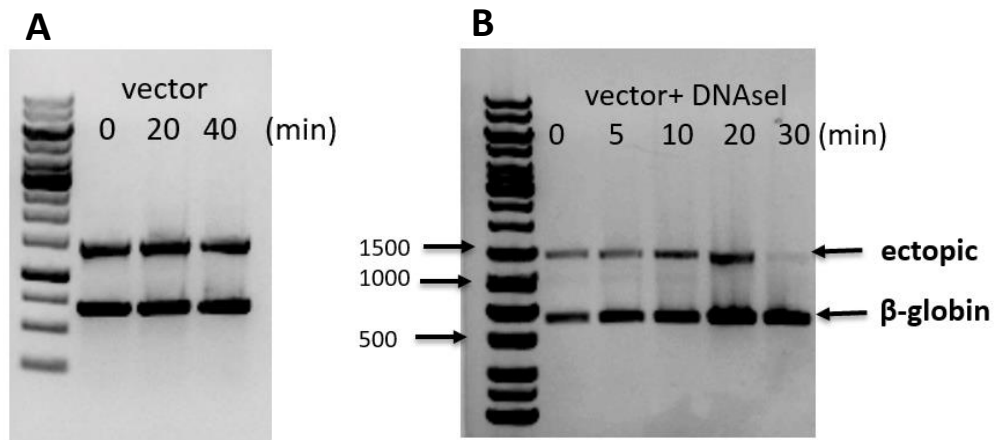
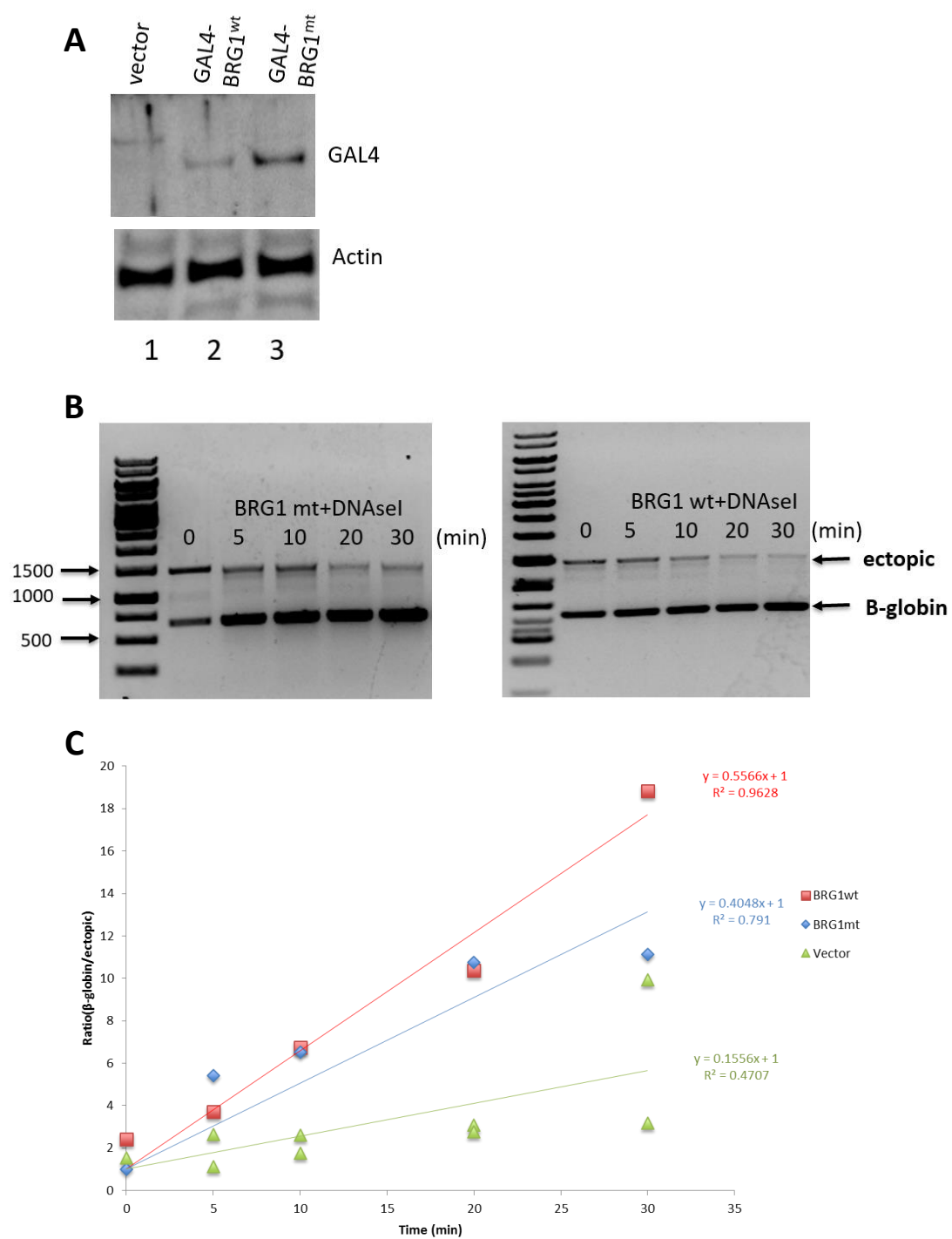


Figure 17. GAL4^{DBD}-BRG1 (both wild type and mutant forms) fusion protein increased the nuclease sensitivity of HeLa/FRT.myc.5'(930)-GAL4 cells.

(A) Western blot with anti-GAL4 antibody of extracts from HeLa/FRT.myc.5'(930)-GAL4 cells transfected with vector only (lane 1), cells expressing GAL4^{DBD}-wild-type BRG1 (lane 2), cells expressing GAL4^{DBD}-BRG1^{K798R} mutant (lane 3).

(B) Genomic DNAs of time-dependent DNase I-treated of GAL4^{DBD}-BRG1^{K798R} mutant-transfected, or GAL4^{DBD}- wild-type BRG1-transfected, HeLa/FRT.myc.5'(930)-GAL4 cells were extracted and purified before PCR amplification and agarose gel electrophoresis.

(C) The β -globin/ectopic ratios of vector-transfected, GAL4^{DBD}- wild-type BRG1-transfected, and GAL4^{DBD}-BRG1^{K798R} mutant-transfected HeLa/FRT.myc.5'(930)-GAL4 cells were scatter-plotted and fitted for linear regressions. GAL4^{DBD}- wild-type BRG1- or GAL4^{DBD}-BRG1^{K798R} mutant transfection was done once, and vector transfection was done two times, and the DNase I hypersensitivity of each treatment is quantified by the slope of each linear regression. Red squares indicate GAL4^{DBD}- wild-type BRG1 transfected DNA samples, blue diamonds indicate GAL4^{DBD}-BRG1^{K798R} transfected DNA samples, and green triangles indicate vector-transfected DNA samples.



IV. DISCUSSION

DNA unwinding element (DUE) is not sufficient to reactivate an inactive replicator:

DUEs have been found in DNA replication origins from prokaryotes to eukaryotes (Benbow et al., 1992; Dobbs et al., 1994; Huang and Kowalski, 1996; Ishimi and Matsumoto, 1994; Liu et al., 2003). In yeast, it has been shown that the binding site for origin recognition complex (ORC) and the easily unwound sequence DUE are two essential components for DNA replication initiation, regardless of the different replication origins.

Our studies have consistently shown that DUEs are essential elements of mammalian DNA replication origins. We demonstrated that deletion of the DUE eliminated origin activity and DUE-B binding at the c-myc replicator (Figure 6, Ghosh et al., 2006). The lost origin activity, DUE-B, and Cdc45 binding can be recovered when the DUEs are replaced with (ATTCT)_{n≥27} repeats from the ATXN10/SCA10 locus (Chowdhury et al., 2010; Liu et al., 2007). Furthermore, GAL4^{DBD} DUE-B was tethered to the GAL4-binding site in place of the ectopic c-myc replicator DUE. However, origin activity and Cdc45 binding were not restored even in the presence of Orc2, Mcm7 and GAL4^{DBD} DUE-B binding (Chen et al., 2013).

Taken together, these results demonstrated that a DUE sequence is essential for c-myc replicator activity in addition to DUE binding. However, it remains unclear whether a DUE sequence alone is sufficient for DNA unwinding and origin activity. To address this question, the chromatin accessibility of the c-myc DUE sequence in the presence of protein tethering was evaluated in three different deletion mutants of c-myc replicator, all of which contain the DUE. DNase I was first used to distinguish the nuclease accessibility of active from inactive genes (Weintraub and Groudine, 1976). DNase I cleaves linker DNA unprotected by histones, thus DNase I can be used to map of nucleosome locations (Simpson 1999). Moreover, DNase I hypersensitivity sites, due to

their exposed state on the chromatin, are often associated with enhancers, promoters, replication origins and other features of DNA activity (Wang and Simpson 2001).

In this study, DNase hypersensitivity assays were used to determine chromatin structure of the ectopic c-myc replicators. β -globin locus was used as an internal control used for DNase digestions because of its chromatin is resistant to DNase I in a time-dependent manner (shown in Figure 11c). Therefore, the increased trend in DNase I sensitivity of the ectopic c-myc locus in comparison to that of the β -globin locus indicates an increase in nuclease accessibility, which correlates with a more open chromatin state.

In this study, GAL4^{DBD}-Cdt1 is the fusion protein used for protein tethering to the GAL4 binding sites fused to the c-myc ectopic replicators. The exogenous protein expression levels might affect the DNase I hypersensitivity in each HeLa/406 derivative cell line. Another concern is that the expression levels of endogenous Cdt1 were approximately five times the expression levels of GAL4^{DBD}-Cdt1 across cell lines (Figure 12a, 14a, and 15a). However, the ratio of GAL4^{DBD}-Cdt1 to GAL4 binding sites are much greater than the ratio of Cdt1 molecules to origins due to GAL4 specificity of the ectopic c-myc locus. Therefore, GAL4^{DBD}-Cdt1 molecules can bind every available GAL4 binding site even if some of them enter the pool of endogenous Cdt1 molecules and bind non-specific origins.

In the presence of GAL4^{DBD}-Cdt1, FRT.myc.5'(930)-GAL4 replicator displayed increased nuclease sensitivity, while shorter forms of c-myc replicators (FRT.myc.5'(607)-GAL4 and FRT.myc.5'(228)-GAL4) had unchanged nuclease sensitivity. These data suggest that additional DNA sequences (up to 930 bp) surrounding the DUE sequence contribute to the opening of nucleosomal packing, which is required for protein binding and origin DNA unwinding, the crucial events leading to DNA replication initiation. In order to determine whether it is the DNA sequence itself or its length which is important for nuclease sensitivity, ectopic replicator containing scrambled DNA with the same length as that of FRT.myc.5'(930)-GAL4 in conjunction to the c-myc DUE can be subjected to DNase I digestion. Furthermore, since DNA unwinding is defective in these new c-myc origin deletion mutants in the presence of

GAL4^{DBD}-Cdt1 expression, we predict that there will be no origin activity at the ectopic mutant c-myc replicators in the presence of GAL4^{DBD} fusion protein tethering.

GAL4^{DBD}-BRG1 increased the nuclease sensitivity of the c-myc DUE in HeLa/FRT.myc.5'(930)-GAL4 cells:

BRG1 is a core catalytic subunit of mammalian SWI-SNF chromatin remodeling complex. BRG1 uses its ATPase activity to break DNA-histone contacts, leading to repositioning of nucleosomes on the chromatin. Recently, BRG1 has been shown to co-localize with DNA replication factors such as ORC, GINS and proliferating cell nuclear antigen on chromatin fibers (Cohen et al., 2010). This novel function of BRG1 suggests that this protein might be involved in origin firings and DNA replication elongation. Taken together, BRG1 functions as a chromatin remodeler, which is not only important for gene transcription but also for efficient replication fork progression. However, the involvement of BRG1 in DNA replication is unclear.

This study attempts to address this question by tethering GAL4^{DBD}-wild-type BRG1 or GAL4^{DBD}- BRG1^{K798R} catalytic dead mutant to the inactivated ectopic FRT.myc.5'(930)-GAL4 c-myc replicator. As a result, GAL4^{DBD}- wild-type BRG1-expressing HeLa/FRT.myc.5'(930)-GAL4 c-myc cells showed the highest increase in DNase I sensitivity, whereas GAL4^{DBD}- BRG1^{K798R}-expressing cells show moderate increase in DNase I sensitivity in comparison to empty-vector treated cells.

Both the wild-type BRG1 and the catalytic dead BRG1^{K798R} mutant have been shown to stimulate BAF155 and cyclin E complex formation (Shanahan et al., 1999). BAF155 is a core subunit of the mammalian SWI-SNF complex, which stabilizes the complex and promotes BRG1 ATPase activity (Phelan et al., 1999; Chen and Archer, 2005). Cyclin E accumulates in late G1 phase, binds to Cdk2, and promotes replication licensing by phosphorylating Cdc6 protein (Mailand and Diffley, 2005). In a Cdk2-independent fashion, cyclin E can also interact with the pre-RC licensing protein Cdt1 on chromatin and facilitates the loading of the Mcm2-7 helicases (Geng et al., 2007).

Therefore, the presence of either GAL4^{DBD}- wild-type BRG1 or GAL4^{DBD}- BRG1^{K798R} might recruit cyclin E to the ectopic c-myc locus, which in turn can facilitate replication licensing (via Cdc6 and Cdt1), as well as Mcm2-7 loading onto the chromatin.

However, only GAL4^{DBD}- wild-type BRG1 retains ATPase activity, which can alter the nucleosome positioning and expose c-myc DNA for nuclease digestion. The data presented here supported the above predictions. Wild-type BRG1 increased DNase sensitivity of the ectopic 5' 930 bp c-myc most significantly, while BRG1^{K798R} mutant increased DNase sensitivity moderately in comparison to empty vector-transfected cells. We hypothesize that GAL4^{DBD}-wild-type BRG1 might be able to recruit other endogenous replication proteins (Cdc45, DUE-B, and ORC) and restore origin activity of the inactivated c-myc origin. Additional experiments, such as chromatin immunoprecipitation and quantitative PCR, are necessary to support this hypothesis.

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